FERMENTATION OF CELLULOSE TO ETHANOL BY *KLEBSIELLA OXYTOCA* CONTAINING CHROMOSOMALLY INTEGRATED GENES ENCODING THE *ZYMOMONAS MOBILIS* ETHANOL PATHWAY

By

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Major Department: Microbiology and Cell Science

Complete enzymatic hydrolysis of cellulose to glucose is generally required for efficient fermentation to ethanol. This hydrolysis requires the combined activities of endoglucanase, exoglucanase, and cellobiase. The Gram-negative bacterium, *Klebsiella oxytoca*, contains the native ability to transport and metabolize cellobiose, minimizing the need for extracellular cellobiase. *K. oxytoca* strain P2 is a recombinant derivative in which the *Zymomonas mobilis pdc* and *adhB* genes have been integrated into the chromosome and expressed, directing the metabolism of pyruvate to ethanol. This organism has been evaluated in simultaneous saccharification and fermentation (SSF) experiments to determine optimal conditions and limits of performance. Temperature was varied between 32°C and 40°C over a pH range of 5.0-5.8 with 100 g liter^{-1} crystalline cellulose (Sigmacell 50, Sigma

Chemical Co., St. Louis, MO) as the substrate and commercial cellulase (Spezyme CE, Genencor, S. San Francisco, CA). An optimum for SSF was observed, with a pH of 5.2-5.5 and temperatures of 32-35°C, which produced over 44 g ethanol $liter^{-1}$ (81-86% of maximum theoretical yield).

Pretreatment of sugar cane bagasse was essential for an SSF process utilizing *K. oxytoca* strain P2 and Spezyme CE. A combination of 20 filter paper units (FPU) of cellulase g^{-1} bagasse, preincubation under saccharification conditions, and additional grinding were required to produce approximately 40 g ethanol $liter^{-1}$. Alternatively, almost 40 g ethanol $liter^{-1}$ was produced with 10 FPU of cellulase g^{-1} bagasse by incorporating a second saccharification step (no further enzyme addition) followed by a second inoculation and short fermentation. In this way, a theoretical ethanol yield of over 70% was achieved with the production of 20 g ethanol per 800 FPU of commercial cellulase.

Escherichia coli strain KO11 may be used to generate ethanol from xylose or hemicellulose hydrolysate while producing recombinant bacterial cellulase for SSF supplementation using *K. oxytoca* strain P2 and Spezyme. Using *E.coli* strain KO11 and plasmid pCT603 containing the *C. thermocellum celD* gene, the amount of fungal cellulase required to reach 70 % of maximum theoretical ethanol yield was decreased to 5 FPU g^{-1} cellulose. These experiments suggest that bacterial cellulase supplementation of exisiting SSF processes may reduce the amount of fungal cellulase needed for adequate ethanol production.

CHAPTER I
GENERAL INTRODUCTION

Ethyl alcohol has been used by the human race since the dawn of history and is thought to be one of the most universally known chemical compounds manufactured (Jacobs, 1950). Produced by the spontaneous fermentation of sugars, it was utilized by ancient civilizations who evolved many types of alcoholic beverage production. The Arabs and Romans actually extracted and concentrated alcohol from fermented material in crude stills. This alcohol was used in the manufacture of perfumes, cosmetics and medicinal agents, and was consumed also as beverages. Ethanol found increasing use as a chemical agent, an ingredient, or a raw material for the production of other commodities, as later civilizations improved upon the purification and distillation processes. With the entrance of the United States into World War II, the alcohol requirements for munitions, synthetic rubber, solvents and thinners and food, increased the demand to unprecedented levels (Jacobs, 1950).

Ethanol may be derived by fermentation processes from three main groups of materials: saccharine materials, where the carbohydrate is present in the form of sugar (molasses fruit, sugar cane, etc.); starch materials (cereal grains,

root crops such as potatoes, etc.); cellulosic or lignocellulosic materials (wood, waste sulfite liquor from paper pulp mills, agricultural residues such as corn cobs, hulls, stover, etc.). Considerable effort was invested in utilizing sawdust and mill waste for ethanol production as early as 1915, using a dilute sulfuric acid process (Jacobs, 1950). Ethanol was produced commercially in the U. S. by this method until the end of World War I, when molasses became a cheap source of readily available substrate.

Fermentation processes utilizing wood wastes intensified abroad, however, since wood waste was more economically available than molasses or grain. By 1941, about 21 foreign plants are reported to have operated on wood wastes. A commercial plant was erected for the development of ethanol from wood wastes in the United States by Defense Plants Corporation, and was operational in 1947 (Jacobs, 1950). However, the further development of this technology ceased with the end of World War II, when the era of inexpensive petrochemical fuels began (Emert et al., 1983).

However, the bioconversion of cellulosics was of interest to the U. S. Army Natick Research and Development Command pertaining to the prevention of microbial degradation of cellulosic materials utilized by the military (Reese, 1976). A team of army scientists isolated the fungus *Trichoderma longibranchiatum (reesei)* in 1943. This organism was responsible for decomposition of military clothing, tents, and

other equipment. The army researchers identified an active cellulase complex that was stable and contained all the components needed to hydrolyze native cellulose. Research was undertaken to examine the applications for the enzyme system from *Trichoderma longibranchiatum*, including roles in food production, energy generation, and waste disposal. A hyper-secreting *T. longibranchiatum* mutant was isolated in 1971, and in 1973 considerable effort was focused on process development for production of cellulases and pretreatment options for various feedstocks (Gracheck et al., 1981).

In 1974, Gulf Oil Chemicals Company undertook extensive research and development to examine processes that could convert cellulose to chemicals. Their objective was to establish a chemical industry that was based on a renewable resource rather than petroleum (Dyess and Emert, 1981). In addition, the subsequent OPEC oil embargo emphasized the importance of this type of industry possibly leading to energy independence.

Another attractive aspect of the lignocellulose-to-ethanol process is that it lends itself to the problems of solid waste management. Waste management issues are becoming increasingly more serious and have reached a critical stage in many areas both in the United States and abroad. More stringent regulatory restrictions concerning ocean dumping and landfill disposal of waste materials have been implemented (Lynd et al., 1991). Utilization of a process that converts

cellulose to ethanol can reduce the amount of material to be discarded and make a useful product simultaneously.

In the United States, most ethanol produced by fermentation is from corn, which is about 80% starch, an α-1,4 linked polymer of glucose that is easily digested by man (Wright, 1989). The starch is hydrolyzed to glucose and then metabolized by the organism to ethanol. Even though this process is cost competitive now, if the ethanol industry were to expand more than 2-fold, the current corn surplus would be consumed and corn prices would rise, which would in turn increase the price of ethanol and eventually limit industry growth (Wright, 1989). Therefore, a more plentiful and less expensive source of carbohydrate is needed as the feedstock.

Cellulosic wastes, agricultural residues, and forage and woody crops are significant renewable resources for the production of fermentable sugars (Becker et al., 1981; Lynd et al., 1991; Barbosa et al., 1992; Beall et al., 1992). The largest reservoir of organic carbon fixed annually by plants is biopolymers of a carbohydrate and polyphenolic nature (Grohmann and Himmel, 1991). This raw material can be obtained at very reasonable costs, and, with the exception of starch, many of these carbohydrate biopolymers cannot provide nutrition for humans (Grohmann and Himmel, 1991).

Currently, no one particular process or technology for lignocellulosic biomass conversion to ethanol has proven itself to be substantially better than the alternatives. The

choice of the pretreatment method to separate the components, the hydrolysis method used to convert the carbohydrates into sugars and the balance between capital and operating costs versus conversion efficiency are some of the tradeoffs involved in the selection of one technology over another (Kerstetter and Lyons, 1991). The main challenge is to develop an efficient and inexpensive process that can use the entire complex structure of lignocellulose: hemicellulose, lignin, and cellulose (Wright, 1989).

Ethanol production by fermentation of lignocellulosic biomass-derived sugars involves a fairly ancient art and a newly developing science. In general, lignocellulose is treated to open the plant wall structure and disrupt lignin-hemicellulose complexes (Grohmann and Himmel, 1991). Hemicellulose is a heterogeneous branched polymer that yields mostly xylose upon hydrolysis, as well as some arabinose, mannose, glucose, galactose, acetic acid and furfural (Ladish et al., 1983; Mehlberg et al., 1980; Eriksson et al., 1990). Hydrolysis of the hemicellulose component is relatively easy, and recent developments in pentose fermentation by recombinant bacteria have improved the outlook for this technology considerably (Ingram et al., 1991; Burchardt and Ingram, 1992; Beall et al., 1992). The difficulties involved in cellulose hydrolysis are the opposite of those for hemicellulose. Fermentation of the cellulose hydrolysis product, glucose, is relatively straightforward, while the actual hydrolysis step

itself is more difficult (Eriksson et al., 1990; Ladish et al., 1983). In its native form, cellulose is composed largely of crystalline fibers in which hydrogen bonds hold the polymer strands together. These fibers are embedded in a matrix of hemicellulose and lignin, which serves to reduce further their accessibility to cellulolytic enzymes (Beguin, 1990). Solvent and mechanical pretreatments increase the accessibility of cellulose to hydrolysis presumably by disrupting the lignin lattice and crystalline structure of the cellulose. Therefore, pretreatment of lignocellulosics improves cellulose conversion (Ladish et al., 1983; Grohmann and Himmel, 1991; Thompson et al., 1992). Pretreatment methods that have been examined for biomass conversion to ethanol include acid pre-hydrolysis, steam explosion, ammonia fiber (freeze) explosion (AFEX), alkali treatment, organic solvents, and radiation (Kerstetter and Lyons, 1991; Wright, 1989; Emert et al., 1983; Holtzapple et al., 1991). Many of the pretreatment methods, in addition to separating the cellulose, hemicellulose, and lignin, also hydrolyze the hemicellulose to xylose, so no further hydrolysis is required. In contrast, cellulose must be converted to glucose or cellobiose for fermentation to ethanol. Lignin, the third major component of lignocellulose, is a large phenolic polymer that cannot be fermented to ethanol but can provide sufficient energy for ethanol recovery (Kerstetter and Lyons, 1991).

The enzymatic hydrolysis of cellulose is particularly attractive due to its selectivity and low impact on the environment (Eriksson et al., 1990; Jeffries, 1988; Grohmann and Himmel, 1991). The complete hydrolysis of cellulose to glucose requires three major classes of enzymes: 1) exoglucanases, which attack nonreducing ends of crystalline cellulose chains; 2) endoglucanases, which degrade amorphous cellulose and may also introduce nicks in crystalline cellulose chains; and 3) ß-glucosidase (cellobiase), which completes the process by degrading cellobiose into glucose monomers (Eriksson, 1990; Enari, 1983). Microorganisms that degrade cellulose are ubiquitous and abundant in nature. These include fungi, bacteria, and actinomycetes. However, relatively few organisms produce the complete extracellular cellulase systems needed to degrade crystalline cellulose efficiently *in vitro* (Coughlan, 1990). The ability to produce extracellular cellulolytic enzymes is widespread among fungi, with *Trichoderma longibranchiatum* being one of the most extensively studied. Culture filtrates from this organism contain each of the major cellulolytic enzymes in a number of forms. Several possible causes of this multiplicity are (a) complexing with other proteins, glycoproteins or polysaccharides; (b) variants of a single gene product as a result of infidelity in post-translational modification, differential proteolysis or glycosylation, and/or interaction with components of the culture broth; and (c) the occurrence

of multiple genes. Examples of all possibilities have been described in the literature (Coughlan, 1990; Enari, 1983). While a multiplicity of each of these three major components exists (Table 2), the mode of action of each general group of enzymes can be summarized for fungal enzymes as follows (Enari, 1983) (Table 1): (a) endoglucanase; (b) ß-glucosidase; (c) cellobiohydrolase. Endoglucanases hydrolyze ß-1,4-glycosidic linkages randomly and do not attack cellobiose. Most reports indicate no action on crystalline cellulose. Endoglucanases hydrolyze cellodextrins, phosphoric-acid-swollen cellulose and substituted celluloses (indicating low specificity). Beta-glucosidase (cellobiase) hydrolyses cellobiose and cello-oligosaccharides to glucose and does not attack cellulose or higher cellodextrins. Cellobiohydrolase (exocellulase) splits off cellobiose units from the nonreducing end of the chain, does not attack substituted celluloses, and hydrolyzes cellodextrins, but not cellobiose. A minor activity, glucan glucohydrolase, whereby glucose units are removed from the nonreducing end of the chain, has been reported by some investigators (Coughlan, 1985).

Most fungal extracellular cellulolytic components are glycoproteins, with varying degrees of glycosylation. Some proposed explanations for the existence of covalently bound carbohydrate include stabilization of protein conformation,

Table 1. Hydrolysis of different substrates by cellulolytic enzymes.

Type of Enzyme	Substrate				
	Crystalline cellulose	CMC	Amorphous cellulose	Cello-tetraose	Cellobiose
Endoglucanase	-(+)	+	+	+	-
Cellobiohydrolase	+	-	+	+	-
Cellobiase	-	-	-	+	+

(Enari, 1983)
CMC, carboxymethylcellulose

protection against proteolysis, and possible roles in substrate recognition or secretion (Coughlan, 1990).

Although the cellulase systems of anaerobic bacteria (Coughlan and Ljungdahl, 1988) and some anaerobic fungi (Wood et al., 1988) exist as large multisubunit complexes, the extracellular components of aerobic fungal systems have generally been considered to be soluble. Complexes found by some researchers were initially presented to be incidental rather than functional (Coughlan, 1985). However, Sprey and Lambert (1983) reported the appearance of complexes in culture filtrates of *T. longibranchiatum*. One complex appeared as a homogeneous band on preparative isoelectric focusing in polyacrylamide gels, and could be partially resolved into its individual components only when treated with urea-octylglucoside and refocused in the presence of urea (Sprey and Lambert, 1983). This complex consisted of six proteins and exhibited endoglucanase, ß-glucosidase, and xylanase activities and appeared to be held in association by remnants of the fungal cell wall. The authors emphasize that after isoelectric focusing in the absence of detergent, homogeneity reflects the purity of a complex and not necessarily of a single protein. These observations suggested to the authors that complex formation in culture filtrates is functional rather than incidental. This conclusion is further supported by the difficulty of separating cellulolytic components by

standard fractionation procedures and the observed synergism between components (Coughlan, 1990).

Extracellular protease production may be required for the release of endoglucanases from fungal cell walls (Eriksson and Pettersson, 1982). There appeared to be six distinct endoglucanases in culture filtrates from *T. longibranchiatum*, perhaps due in part to proteolytic modification (Shier-Neiss and Montenecourt, 1984).

The *T. longibranchiatum* genes encoding cellobiohydrolases (CBH I, CBH II), and endoglucanases (EG I, EG II) have since been cloned and sequenced (Knowles et al., 1988). Differences between individual endoglucanases with respect to absorption and desorption on crystalline cellulose suggest that individual isoenzymes perform unique functions in cellulolytic degradation (Coughlan, 1990). One rationale for this observation was proposed by Coughlan and collegues, whereby two endoglucanases and two cellobiohydrolases, in each case differing in stereospecificity, would be required to act upon the stereospecifically different glycosidic linkages in the cellulose (Figure 1; Coughlan, 1990). It was reported in 1954 (cited in Reese, 1974) that the amount of reducing sugar liberated from cellulose by the total fungal culture filtrate was greater than the sum of the amounts released by the individual fractions. Many investigators have since confirmed this observation. This so-called endo-exo synergism has been interpreted by many investigators to suggest that neither type

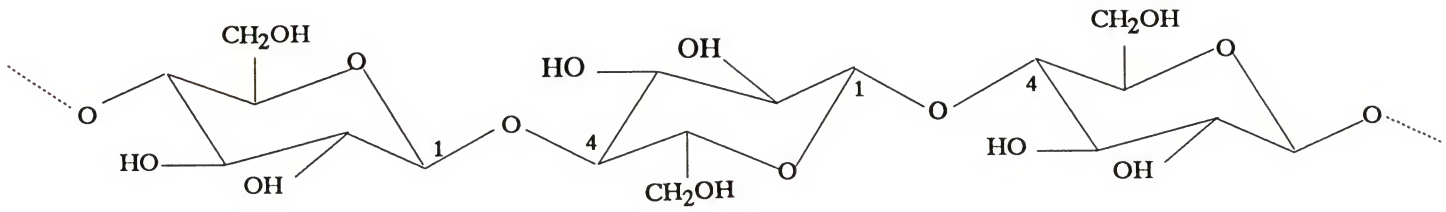


Figure 1. Segment of a cellulose chain showing the ß-1,4-linked glucosyl residues rotated 180° with respect to the adjacent residue. This arrangement causes the linkages to be sterically different to an enzyme approaching the crystalline substrate (Coughlan, 1990).

of enzyme alone can effect extensive hydrolysis of crystalline cellulose (Coughlan, 1990). There is considerable debate as to the exact mechanism(s) of this synergism and to some extent, the differences between the observations by the various groups may reflect the use of different substrates, purification and assay procedures. Recently, Wood et al. (1989) have shown that electrophoretically homogeneous cellobiohydrolase fractions from *T. longibranchiatum* are contaminated with endoglucanase activity. Considerable progress in elucidating the reaction mechanisms of the cellulases has been made. However, much work remains to be completed before a detailed hydrolysis mechanism will emerge.

Another consideration involves the participation of oxidative mechanisms in the breakdown of cellulose. Reports have shown the involvement of oxidative steps in the degradation of cellulose by brown-rot fungi, which do not produce exocellulases but do produce endocellulases and significant amounts of peroxide (Coughlan, 1990). It was proposed that by oxidizing the sugar residues, peroxide in the presence of iron would disrupt the structure of the cellulose and allow attack by the endoglucanases (Koenigs, 1972). Culture filtrates from *T. longibranchiatum* contain a small molecular iron-containing "microfibril generating factor", which generates short fibers from cellulose and is said to act synergistically with endo- and exo-glucanases in solubilizing crystalline substrate (Griffin et al., 1984).

Vaheri (1983) reported that in the initial stages of cotton hydrolysis by *T. longibranchiatum*, short, insoluble fibers appear along with gluconic and cellobionic acids. Coughlan (1990) presumed that the immediate products of an oxidation step would be lactones that would undergo spontaneous hydrolysis to yield the free acids observed. This reaction rate would be increased by the presence of lactonases, which have been found in *A. niger* commercial preparations (Bruchman et al., 1987).

The participation of cellobiose: quinone oxidoreductases (CBQs) and cellobiose oxidases/dehydrogenases has been characterized in *Phanerochaete chrysosporium* (Westermark and Eriksson, 1975). Acting as a link between lignin and cellulose degradation in the white rot fungi, they catalyze the oxidation of cellobiose to cellobionic acid using quinone or phenoxy radicals arising from the breakdown of lignin as the acceptor (Eriksson, 1988). Repolymerization is also prevented by reduction of the laccase-derived phenoxy radical intermediates of lignin degradation (Eriksson, 1988). Cellobiose oxidases are also considered to be a part of the cellulase system of *T. longibranchiatum* since the breakdown of cotton by culture filtrates in a nitrogen atmosphere was only half that observed when reactions were carried out in air or oxygen (Eriksson et al., 1974). Coughlan (1990) proposed that since the cellobiose-oxidizing enzymes are extracellular, they may utilize iron or other transition metals present in the

medium as electron acceptors (and/or quinone or oxygen in the appropriate cases).

Coughlan and Ljungdahl (1988) have proposed a model for the degradation of cellulose and emphasized that it be viewed as a continuum in the conversion of the crystalline polymer to lower molecular weight soluble products (Figure 2). The extent of the involvement of hydrolases, or oxidative reactions, or both, as well as short fiber formation, segmentation, and destratification, are matters of debate and speculation at the moment and are not necessarily mutually exclusive. Also, while pure celluloses are used as model substrates, the physical structure of the substrate is not yet completely defined (Ladish et al., 1983). The hydrolysis reaction is heterogeneous, containing a soluble catalyst, an insoluble substrate and products that are both. Intrinsic kinetic data on absolutely pure protein preparations are lacking, therefore it is difficult to separate kinetic from diffusional effects (Ladish, 1983; Coughlan, 1990). It is generallly agreed that much of this interaction is synergistic, although given the overall complexity of the system, details of the mechanisms whereby the synergism occurs are in dispute.

Although ample experimental evidence exists in the literature indicating that properly treated plant cell wall cellulose fibers can be completely hydrolyzed to sugars by potent cellulase preparations, there are many deficiencies in

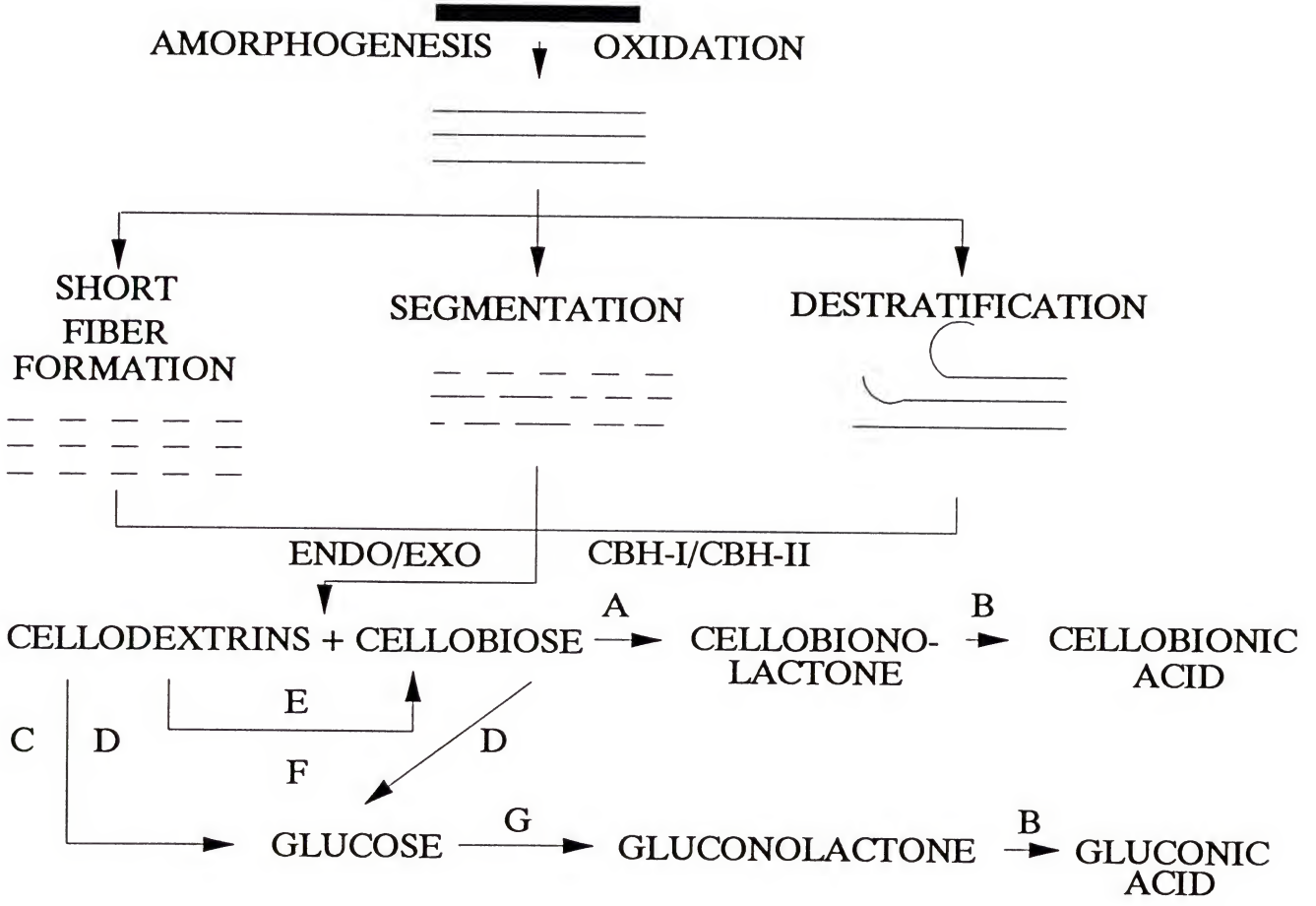


Figure 2. Mechanism of cellulose degradation by fungi proposed by Coughlan and Ljundahl, 1988. The lettered enzymes are: A, cellobiose oxidase/dehydrogenase; B, lactonase; C, exoglucohydrolase; D, ß-glucosidase; E, endoglucanase; F, exocellbiohydrolase; G, glucose oxidase.

commercially available enzyme preparations. Cellulolytic enzymes at each stage are inhibited by the accumulation of products, impeding saccharification (Chahal, 1991; Jeffries, 1988; Mitchell et al., 1991). Cellobiose and glucose are significant product inhibitors. Cellobiose inhibits exocellulase and cellobiase activities, while glucose inhibits the cellotriose hydrolyzing activities of both the endo- and exoglucanase (Ladish et al., 1983; Jeffries, 1988). Commercial cellulases are typically limited by cellobiase instability or the lack of sufficient activity (Chahal, 1991; Mitchell et al., 1991). Saccharification remains incomplete in most cases even with added cellobiase due in part to the accumulation of glucose. Significant advances in the area of enzyme catalyzed saccharification of cellulose have been made in the last 20 years including development of the simultaneous saccharification and fermentation concept as a way to moderate product inhibition (Takagi et al., 1977).

Concerns about dependence on foreign crude oil coupled with increased interest in preservation of the environment have rekindled interest in cellulosic biomass as a source of ethanol for fuel. Few substitutes currently exist for petroleum based transportation fuels, and the United States is vulnerable, both strategically and economically, to disruptions in supply. Ethanol from biomass is a renewable fuel that can be blended with or substituted for gasoline. When produced from lignocellulosics, ethanol generation serves

as a constructive means of recycling carbohydrates in agricultural and industrial waste streams (Becker et al., 1981; Lynd et al., 1991; Kerstetter and Lyons, 1991).

CHAPTER II
FERMENTATION OF CELLULOSE TO ETHANOL BY *KLEBSIELLA OXYTOCA* CONTAINING CHROMOSOMALLY INTEGRATED *ZYMOMONAS MOBILIS* GENES

Introduction

In 1976, a method was patented with yeast as the biocatalyst that prevented glucose accumulation by combining the saccharification and fermentation steps, termed the simultaneous saccharification and fermentation process (SSF) (Blotkamp et al., 1978; Gauss et al., 1976; Takagi et al., 1977). The SSF process represented a significant improvement and was demonstrated in a pilot plant (Emert et al., 1983). However, productivity remained limited by saccharification. Though expensive, productivity can be improved by increasing the level of cellobiase and other cellulases (Desrochers et al., 1981; Freer and Detroy, 1983; Sternberg et al., 1977). The discovery of cellobiose-fermenting yeasts (Barnett, 1976; Blondin et al., 1982; Freer and Detroy, 1983; Maleszka et al., 1982) represented a further improvement by eliminating the need for supplemental cellobiase (Freer, 1991; Spindler et al., 1992; Wyman et al., 1986).

Recent studies have employed genetically engineered Gram-negative bacteria to produce ethanol from sugars with high efficiency by introducing the *Zymomonas mobilis* genes encoding alcohol dehydrogenase and pyruvate decarboxylase

(Beall et al., 1991; Ingram et al., 1991; Ohta et al., 1991ab). Ethanologenic recombinants of *Klebsiella oxytoca*, a soil organism that is particularly abundant in cellulosic waste streams from the pulp and paper industry (Grimont et al., 1991), exhibit the native ability to metabolize xylo-oligosaccharides (Burchhardt et al., 1992) and gluco-oligosaccharides (Al-Zaag, 1989; Wood and Ingram, 1992). SSF fermentations with this organism would be expected to promote saccharification by continuously removing glucose, cellobiose, and cellotriose. However, as with the cellobiose-utilizing yeasts (Spindler et al., 1992; Wyman et al., 1986), pH and temperature optima for commercial cellulases are quite different from those for the biocatalysts. Commercial cellulases, derived from *Trichoderma longibranchiatum (reesei)*, function optimally at pH 4.5-5.0 and 50°C (Chahal, 1991; Genencor International, S. San Francisco, CA). In contrast, *K. oxytoca* is similar to *E. coli* where fermentation optima with glucose (and xylose) were reported to be pH 6.0 (Beall et al., 1991).

In this study, we have investigated the optimal conditions for an SSF process using *K. oxytoca* strain P2 (Wood and Ingram, 1992) as the biocatalyst.

Materials and Methods

Bacterial Strain

Klebsiella oxytoca strain P2 has been previously described (Wood and Ingram, 1992). In this recombinant, the

Zymomonas mobilis genes encoding alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) have been integrated into the *pfl* gene within the chromosome of *Klebsiella oxytoca* strain M5A1 (Ohta et al., 1991a). Strain P2 was maintained on Luria broth (containing per liter 20 g of glucose, 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 40 mg of chloramphenicol) solidified with 1.5% agar.

Preparation of Inocula for Fermentations

Cells were inoculated from a single colony to 500-ml flasks containing 200 ml of Luria broth with 50 g glucose $liter^{-1}$. Cultures were incubated for 24 hours at 30°C without agitation. Cells were harvested by centrifugation (6000g, 10 minutes, 4°C) and used to inoculate SSF fermentations at an initial cell density of 330 mg dry weight $liter^{-1}$.

Fermentation Experiments

Fermentations were conducted in modified 500-ml Fleakers containing 350 mL of broth as previously described (Beall et al., 1991). Glucose was replaced by 100 g $liter^{-1}$ of highly crystalline, purified cellulose (Sigmacell 50, St. Louis, MO), unless otherwise specified. Complex nutrients (2X) were sterilized by autoclaving at 121°C for 15 minutes. Sigmacell 50 cellulose was similarly autoclaved in distilled water. Concentrated hydrochloric acid was used to adjust the intial pH. A 2 M solution of KOH was used to maintain pH during fermentation.

Spezyme CE cellulase was provided by Genencor International and was reported to contain 103 filter paper units (FPU) ml^{-1}. It was sterilized by filtration prior to use and added at a final concentration of 25 FPU g^{-1} cellulose at the time of inoculation, unless otherwise specified.

Analyses

Samples were removed for the determination of ethanol concentration (g $liter^{-1}$) by gas-liquid chromatography (Beall et al., 1991). Ethanol yields were corrected for dilution by the addition of base during fermentations and computed on the basis of total cellulose initially present. No corrections were made for unused carbohydrate or for the production of cell mass. The maximum theoretical yield is 0.568 g ethanol g^{-1} cellulose. Maximum volumetric productivity was estimated from the initial 24 hour period. All results represent averages from two or more fermentations.

Results

Effects of Temperature and pH

Figures 3-10 show SSF fermentations containing 100 g Sigmacell 50 cellulose $liter^{-1}$ and 25 FPU cellulase g^{-1} of cellulose. At the temperature closest to that of the fermentation optimum for the organism, 32°C, maximum ethanol values were achieved for intermediate pH conditions by 120 hours (Figure 3). There was considerable difference in the amount of base consumed to maintain fermentation pH, with the most base consumed at the highest pH (pH 5.8, 132 mmoles)

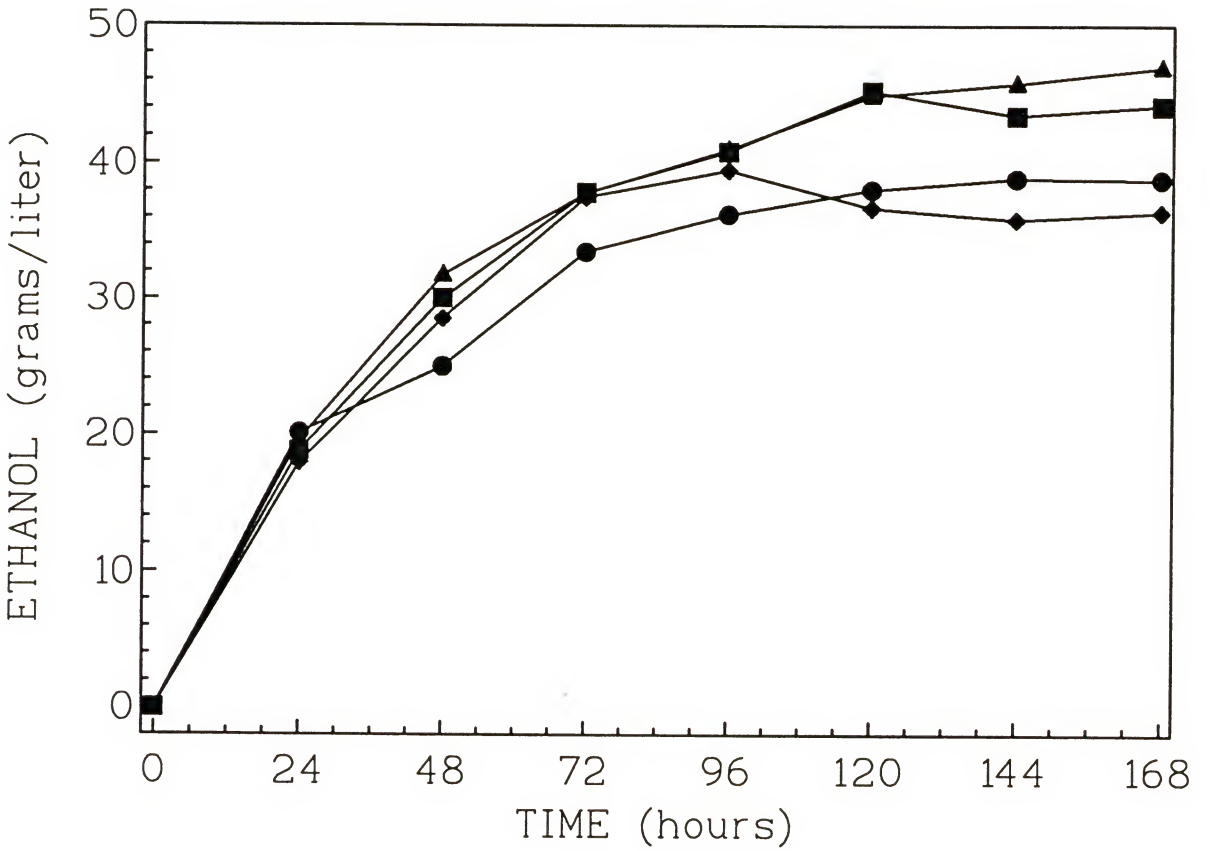


Figure 3. Ethanol concentration during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 32°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

(Figure 4). Figures 5-10 show similar plots for the other temperatures investigated. Table 2 and Figure 11 summarize the effects of temperature and pH. A relatively broad optimal range was observed. The two conditions producing the highest levels of ethanol were pH 5.5 at 32°C (47.0 g ethanol $liter^{-1}$) (Figure 3) and pH 5.2 at 35°C (46.8 g ethanol $liter^{-1}$) (Figure 5). Near equivalent levels of ethanol (45.1 g ethanol $liter^{-1}$) were also produced at 32°C and pH 5.2 (Figure 3). All pH values tested at 35°C and 37°C resulted in 42 g ethanol $liter^{-1}$. At 40°C, less than 40 g ethanol $liter^{-1}$ were produced regardless of pH (Figure 9). Although fermentations were conducted for 168 h, the time required to reach the maximum concentration of ethanol varied between 120 and 144 h for most conditions.

Ethanol reached 85.6% of theoretical yield at 32°C and pH 5.5 and 83.6% at 35°C and pH 5.2 (Table 2). These are equivalent to the production of 0.486 and 0.475 g ethanol g^{-1} cellulose, respectively, after correction for dilution by base. All pH values examined at 35°C exceeded 77% of the theoretical yield. All other conditions tested exceeded 70% of the theoretical yield except low pH at 40°C.

Considerable difference was observed in the amount of base required to maintain pH during fermentation (Figure 11B). In general, more base was needed at elevated temperatures (37°C and 40°C) (Figures 8 and 10) and at higher pH conditions. Fermentations above pH 5.0 at 40°C and at pH 5.8 at 32°C

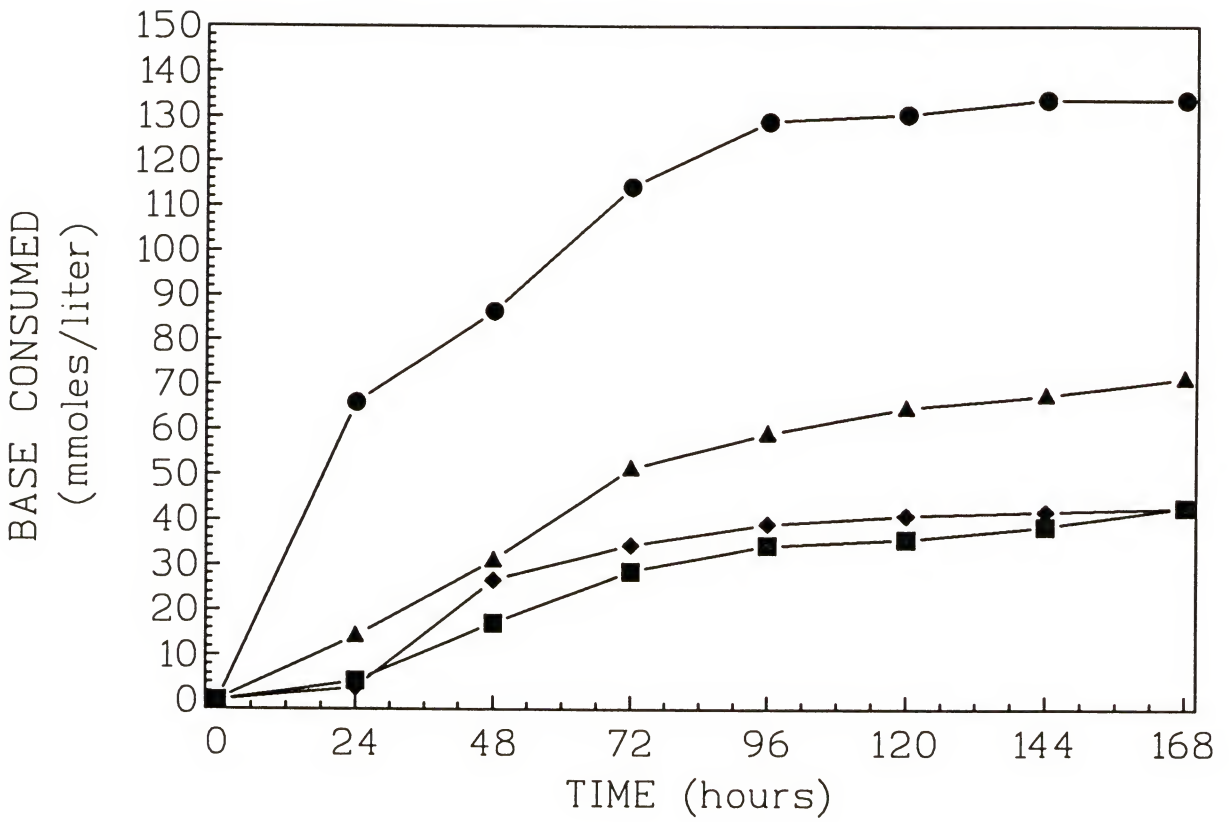


Figure 4. Base consumption during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 32°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

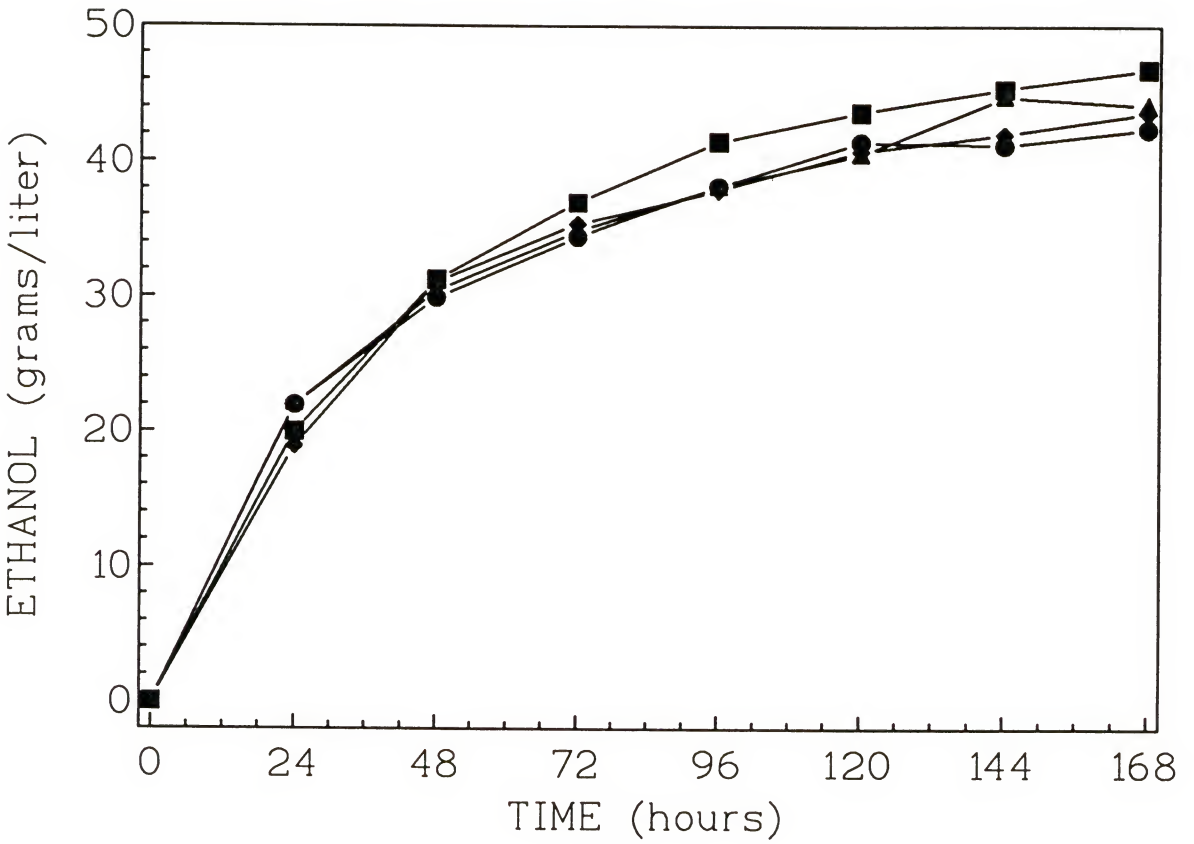


Figure 5. Ethanol concentration during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 35°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

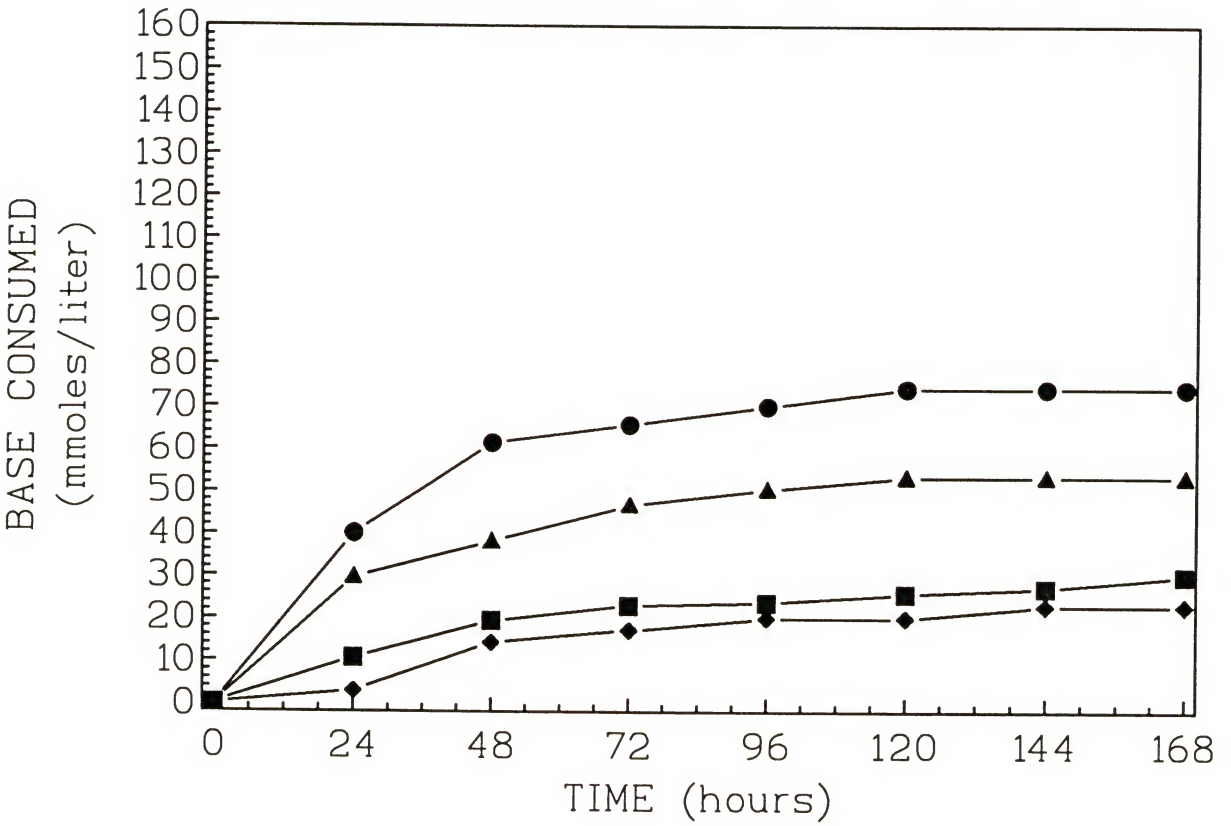


Figure 6. Base consumed during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 35°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

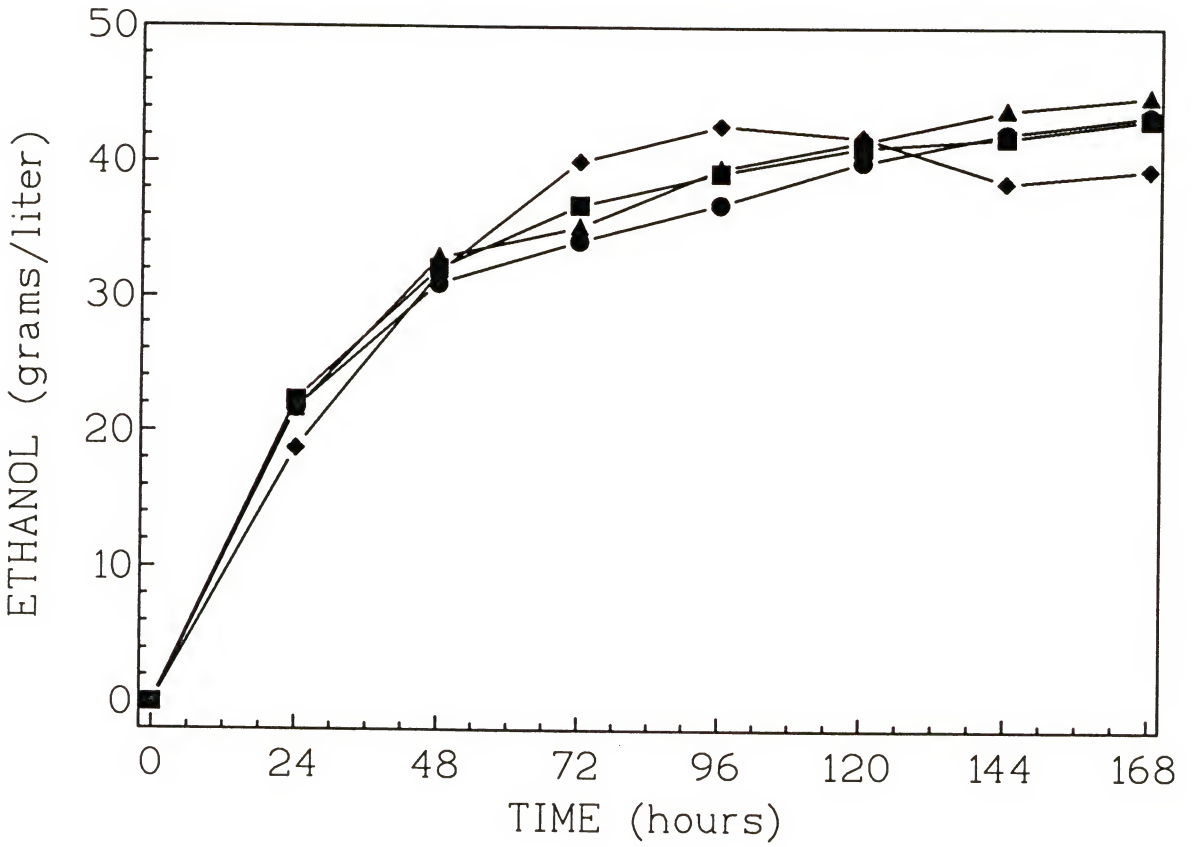


Figure 7. Ethanol concentration during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 37°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

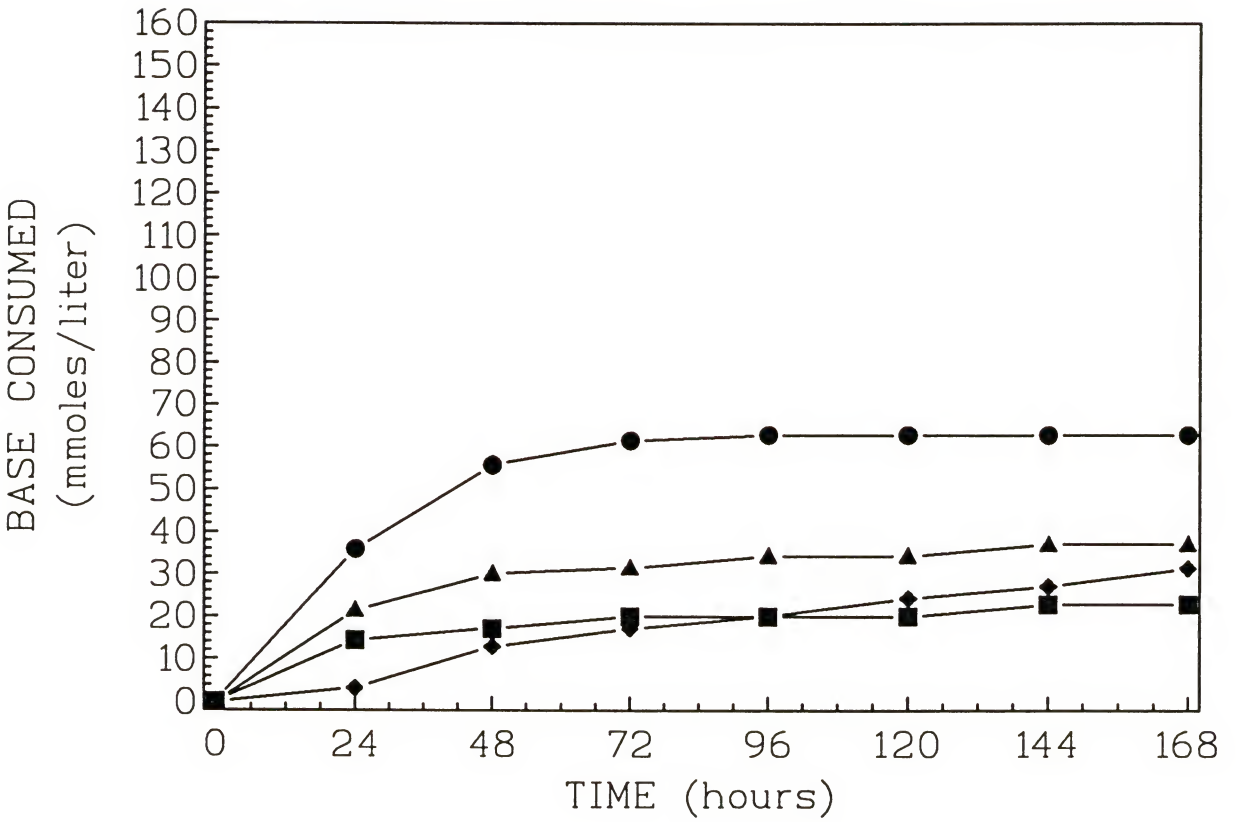


Figure 8. Base consumed during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 37°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

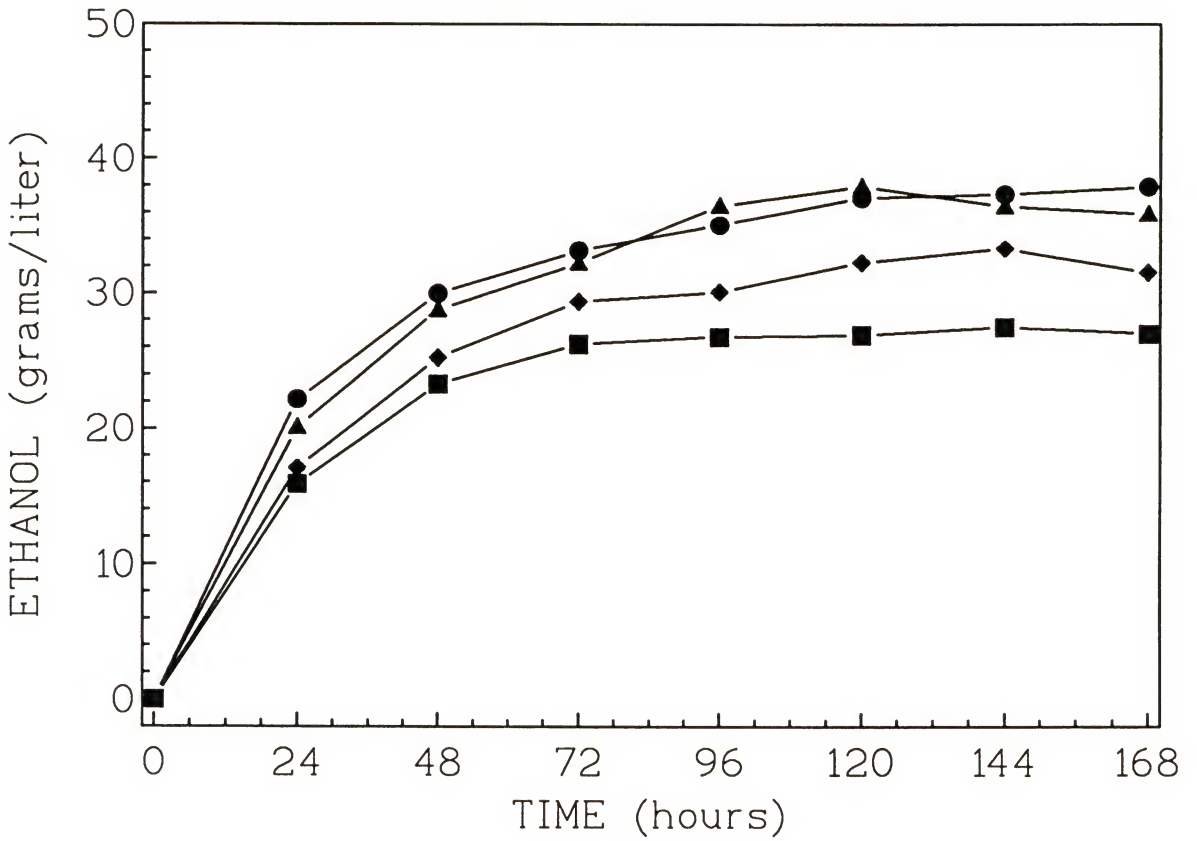


Figure 9. Ethanol concentration during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 40°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

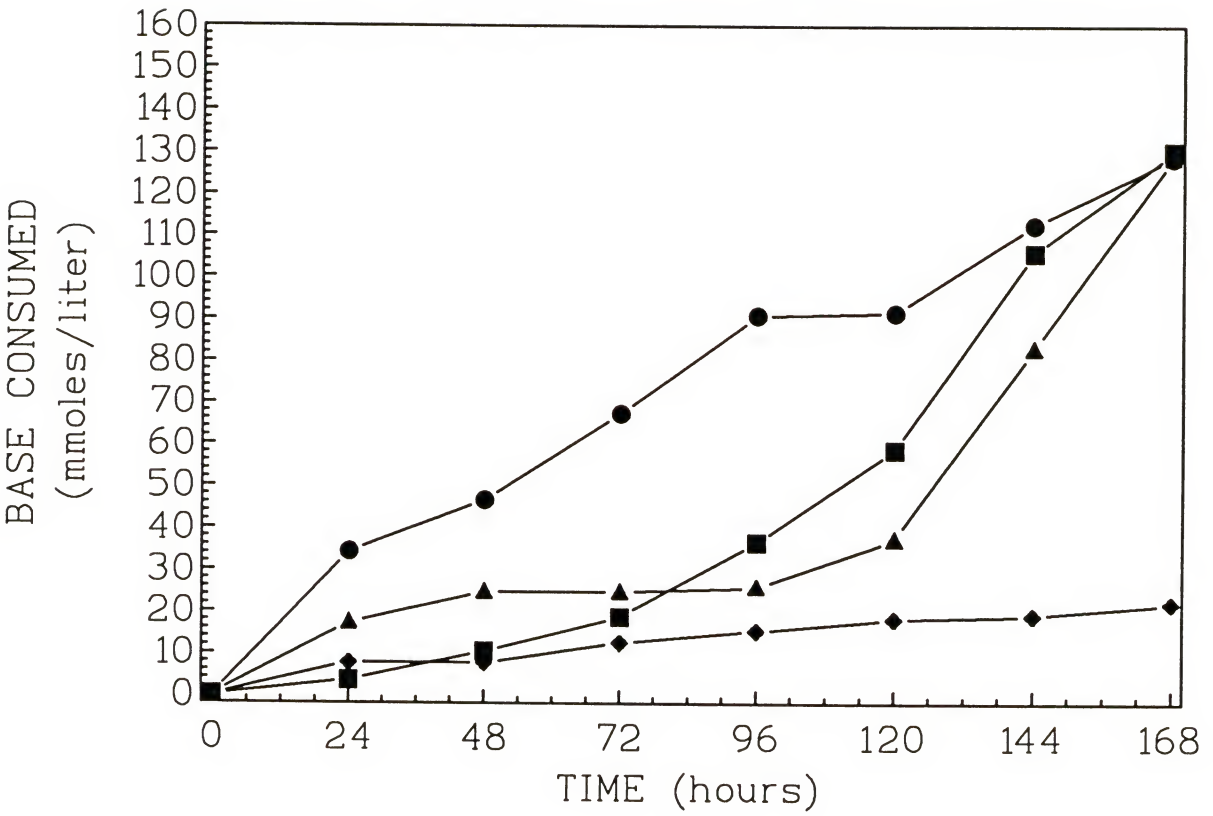


Figure 10. Base consumed during simultaneous saccharification and fermentation of Sigmacell 50 cellulose (100 g liter^{-1}) with Spezyme CE cellulase (25 FPU g^{-1} cellulose) at 40°C, pH 5.0-5.8, by *Klebsiella oxytoca* strain P2. Symbols: ♦, pH 5.0; ■, pH 5.2; ▲, pH 5.5; ●, pH 5.8.

Table 2. Summary of fermentation performance under varying temperature and pH conditions with 100 g Sigmacell cellulose liter^{-1} and 25 FPU Spezyme CE cellulase g^{-1} cellulose.

Fermentation Conditions	Ethanol					
	Maximum concentration[a](Time)[b]	Yield[c]	Theoretical[d] (%)	Volumetric[e] productivity	Base[f] consumed	Time[g] 4% ethanol
32°C						
pH 5.0	39.4 (96 h)	0.402	70.1	0.656	39.0	NA
5.2	45.2 (120 h)	0.460	81.0	0.604	35.7	96
5.5	47.0 (144 h)	0.486	85.6	0.583	71.4	90
5.8	38.9 (120 h)	0.419	73.8	0.521	156.2	NA
35°C						
pH 5.0	43.6 (168 h)	0.441	77.6	0.625	22.9	114
5.2	46.8 (144 h)	0.475	83.6	0.670	30.0	87
5.5	44.7 (144 h)	0.453	80.8	0.625	53.3	114
5.8	42.2 (120 h)	0.440	77.4	0.604	74.3	112
37°C						
pH 5.0	42.7 (96 h)	0.432	76.0	0.667	20.0	72
5.2	43.3 (144 h)	0.438	77.1	0.667	22.9	102
5.5	45.0 (144 h)	0.458	80.6	0.667	37.1	114
5.8	43.6 (168 h)	0.449	79.1	0.646	62.9	120
40°C						
pH 5.0	33.3 (120 h)	0.337	59.3	0.521	19.0	NA
5.2	27.5 (72 h)	0.290	51.0	0.479	105.7	NA
5.5	37.9 (120 h)	0.386	68.0	0.604	128.6	NA
5.8	37.9 (120 h)	0.404	71.1	0.625	128.6	NA

[a] Maximum ethanol concentration (g liter^{-1}).

[b] Time required to reach maximum ethanol concentration (hours).

[c] Ethanol yield per g of added cellulose (g g^{-1}).

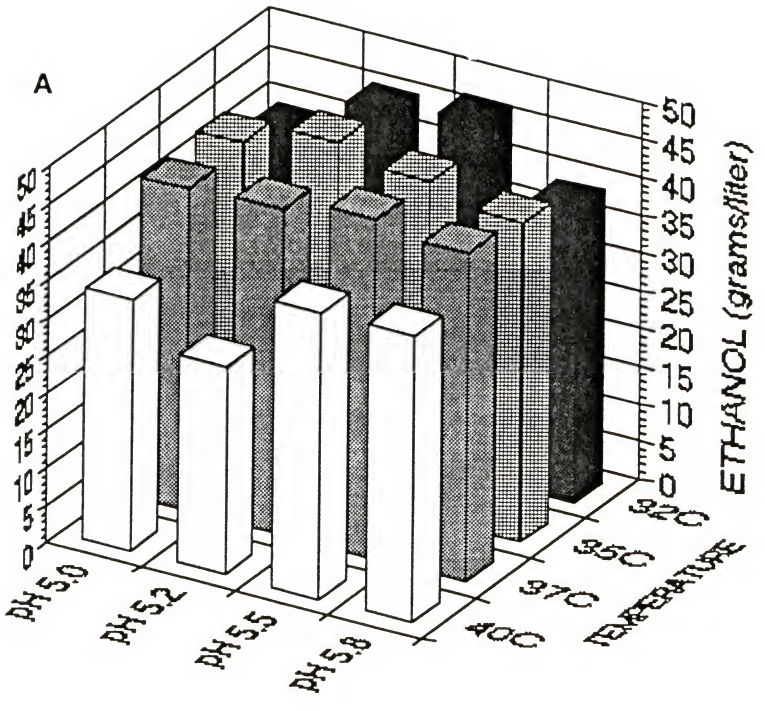
[d] Ethanol yield as a percentage of the theoretical maximum value.

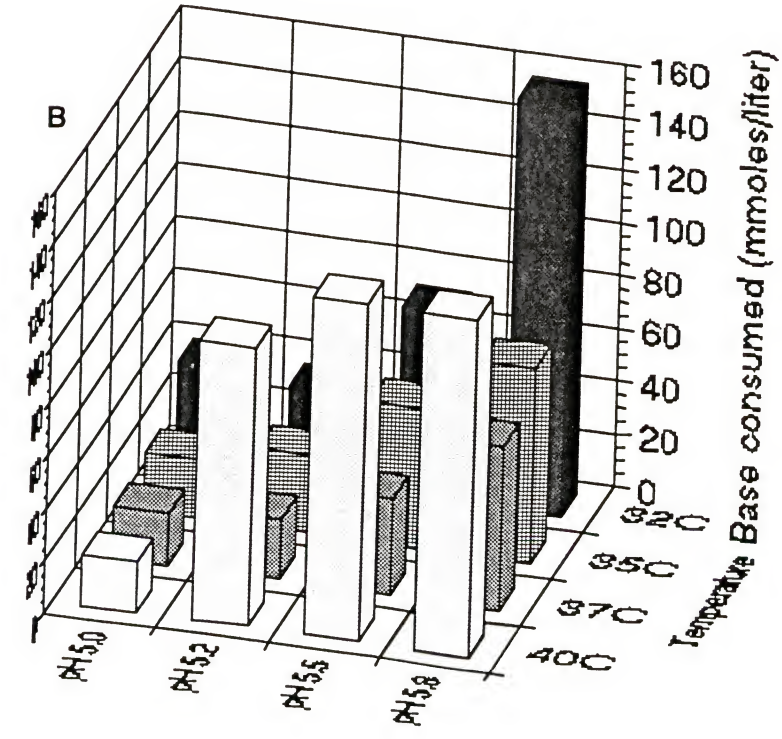
[e] Volumetric productivity (g ethanol liter^{-1} per h) measured during the initial 24 hours of fermentation.

[f] Base consumed during fermentation (mmoles KOH liter^{-1}).

[g] Time required to reach 40 g ethanol liter^{-1} (NA=did not reach this concentration during the course of the 7-day fermentation).

Figure 11. Summary of the effects of temperature and pH on the conversion of cellulose to ethanol by SSF using *K. oxytoca* strain P2. All fermentations contained Sigmacell 50 cellulose (100 g liter^{-1}) and Spezyme CE cellulase (25 FPU g^{-1} cellulose). A. Maximum ethanol concentration. B. Base consumed to maintain pH.


A
ETHANOL (grams/liter)
0
5
10
15
20
25
30
35
40
45
50
pH 5.0
pH 5.2
pH 5.5
pH 5.8
32C
35C
37C
40C
TEMPERATURE


B
Base consumed (mmoles/liter)
0
20
40
60
80
100
120
140
160
pH 5.0
pH 5.2
pH 5.5
pH 5.8
32C
35C
37C
40C
Temperature

consumed large amounts of base. Under optimal conditions for ethanol production (pH 5.2 and 35°C; pH 5.5 and 32°C), 30.0 mmoles KOH liter^{-1} and 71.4 mmoles KOH liter^{-1} were required to maintain pH (Figures 6 and 4, respectively).

The time required to reach 40 g ethanol liter^{-1} was used as a comparative measure of fermentation performance under different conditions. Surprisingly, fermentation conditions with the highest ethanol yields (32°C at pH 5.5; 35°C at pH 5.2) required 87 to 90 h to reach 40 g ethanol liter^{-1} while this level was achieved in less than 72 h at 37°C and pH 5.0. In general, fermentations at 37°C exhibited the highest initial volumetric productivities (Table 2) although final yields were lower than under optimal conditions. Fermentations carried out at 40°C and pH 5.0-5.8, at 32°C and pH 5.0, or 32°C at pH 5.8 did not achieve 40 g ethanol liter^{-1} during 168 h. Low pH fermentations at 40°C exhibited the most variability in base consumption and ethanol yield, indicating that these conditions approach the environmental extremes for ethanol production by strain P2.

Effects of Enzyme Concentration. To determine the minimum amount of enzyme required for acceptable ethanol yields, enzyme loadings of 2, 5, 10, 15, 20, and 25 FPU g^{-1} cellulose were examined under optimal conditions (35°C, pH 5.2) with 100 g cellulose liter^{-1} (Figure 12). An enzyme loading of 10 FPU g^{-1} cellulose appeared to approach saturation since ethanol concentrations obtained from higher loadings (15, 20 and 25

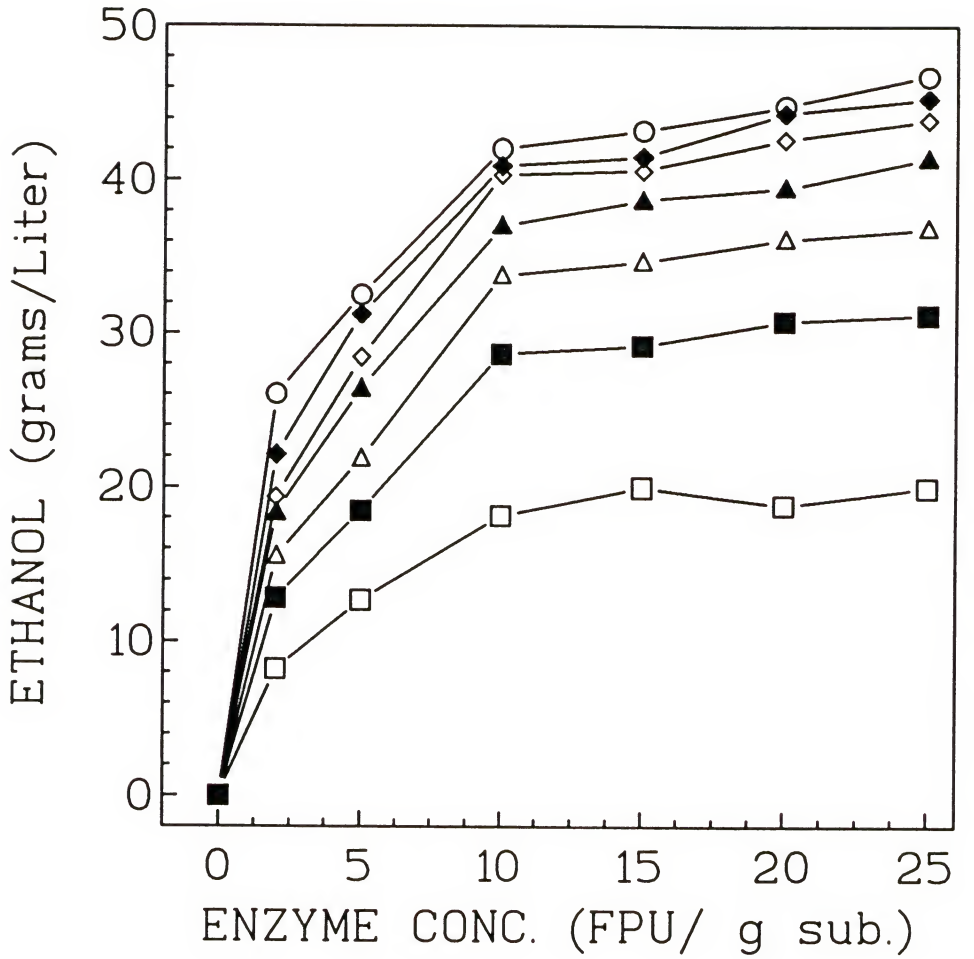


Figure 12. Effect of cellulase concentration (Spezyme CE) on ethanol production by *K. oxytoca* strain P2 during SSF. All fermentations contained Sigmacell 50 (100 g liter^{-1}) and were conducted at pH 5.2 and 35°C. Symbols: □, 24 h; ■, 48 h; △, 72 h; ▲, 96 h; ◇, 120 h; ◆, 144 h; ○, 168 h.

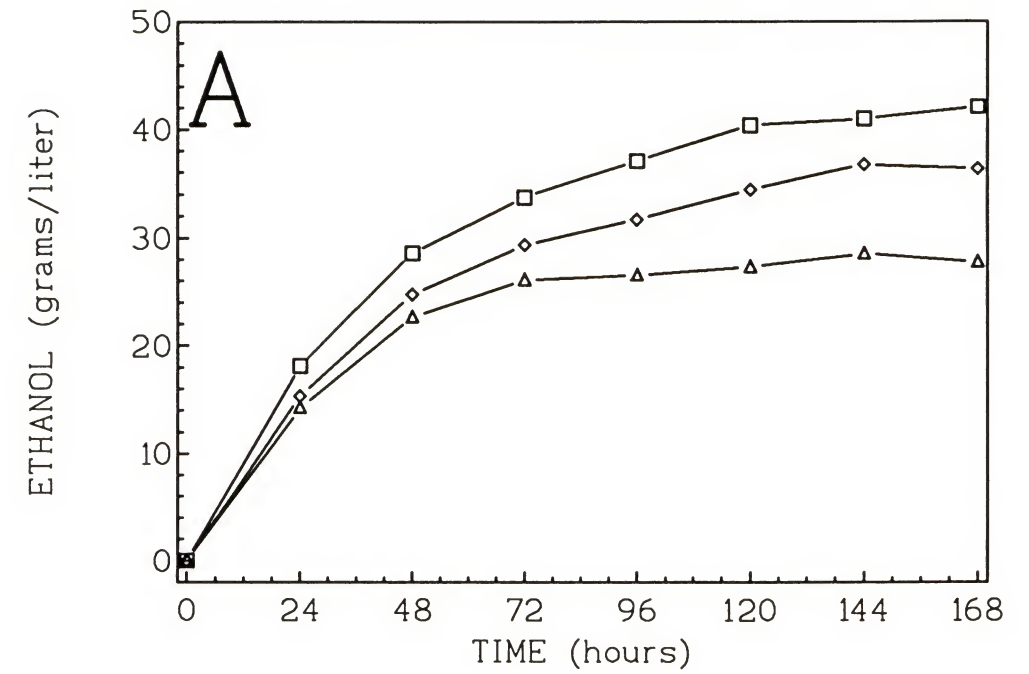
FPU g^{-1} cellulose) resulted in only modest increases in ethanol (42.1 g $liter^{-1}$ versus 43.3 g $liter^{-1}$, 44.9 g $liter^{-1}$, 46.8 g $liter^{-1}$, respectively). Base requirements to maintain pH were not drastically affected by enzyme loading (30.0 mmoles KOH $liter^{-1}$ at 25 FPU to 48.6 mmoles KOH $liter^{-1}$ at 2 FPU). Ethanol yields ranged from 83.6% (25 FPU g^{-1} cellulose) to 46.8% (2 FPU g^{-1} cellulose) of the theoretical maximum. With an enzyme loading of 10 FPU g^{-1} cellulose, 40 g ethanol $liter^{-1}$ (70% of the theoretical maximum) was obtained in less than 120 h.

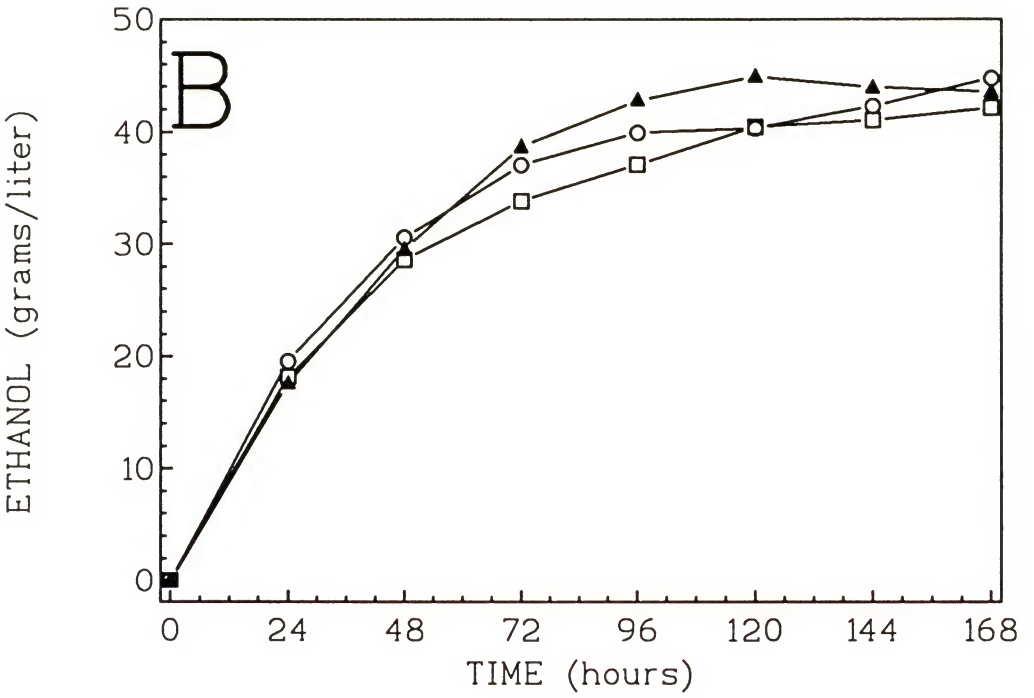
Effects of Cellulose Concentration

The effects of different cellulose concentrations on ethanol yields at a constant loading of cellulase, 10 FPU g^{-1} cellulose have been examined (Figure 13A). Initial rates of ethanol production increased with increasing substrate levels. Maximal yields for cellulose concentrations of 60, 80, and 100 g $liter^{-1}$ were 0.49, 0.47, and 0.43 g ethanol g^{-1} cellulose, respectively.

The rates of ethanol production declined progressively during the saccharification and fermentation process. As the cellulosic substrate levels declined from 100 g $liter^{-1}$, for example, cellulase activity may be limited by substrate availability. Previous experiments had shown that in many cases ethanol values approached or exceeded 20 g $liter^{-1}$ after 24 hours. Thus approximately 40 g $liter^{-1}$ of soluble products

Figure 13. Effect of cellulose concentration (Sigmacell 50) on ethanol production during SSF at pH 5.2 and 35°C. A. Cellulose concentration was varied with a constant cellulase loading of 10FPU g^{-1} cellulose. Symbols: ▵, 60 g $liter^{-1}$; ◊, 80 g $liter^{-1}$; □, 100 g $liter^{-1}$. B. Cellulose concentration was varied with a constant level of cellulase (1000 FPU $liter^{-1}$): □, 100 g cellulose $liter^{-1}$ present at the start of fermentation; ▲, 100 g cellulose $liter^{-1}$ present initially to which 40 g cellulose $liter^{-1}$ was added after fermentation for 24 hours; ○, 140 g cellulose $liter^{-1}$ present at the start of fermentation.


A
ETHANOL (grams/liter)
TIME (hours)
0
10
20
30
40
50
0
24
48
72
96
120
144
168


B
ETHANOL (grams/liter)
TIME (hours)
0
10
20
30
40
50
0
24
48
72
96
120
144
168

have been produced from cellulose by enzymatic hydrolysis and fermented during this initial period.

To test the hypothesis that this decline in rate of ethanol production is due in part to a reduction in substrate concentration (Figure 13B), two experiments were performed. In one, an additional 40 g cellulose liter^{-1} (no additional cellulase) was added after 24 hours. In the other, the extra 40 g cellulose liter^{-1} was added initially. With supplemental cellulose after 24 h, 42.81 g ethanol liter^{-1} was reached after 96 hours as compared to 37.00 g ethanol liter^{-1} with only the initial loading of 100 g cellulose liter^{-1}. Slightly lower yields were obtained when the full 140 g cellulose liter^{-1} was added at the beginning. This decrease could be attributed to reduced mixing.

Supplemental cellulose prolonged the initial rapid rate of ethanol production and resulted in a higher final ethanol concentration. The time required to reach maximal ethanol was reduced by 2 days (28%) and 40 g ethanol liter^{-1} was achieved in 78 h. The effective cellulase loading in these experiments is 7.2 FPU g^{-1} cellulose. The final ethanol yield achieved with this high level of cellulose may be limited by the ethanol tolerance of the organism.

Effects of Incubation with Cellulase Prior to Fermentation

Since the optimal conditions for cellulase activity are more extreme than those tolerated by the fermenting organism, we have also examined the effects of a 12 h pre-incubation of

cellulose with cellulase under conditions which promote maximal enzymatic activity (50°C and pH 5.0) prior to fermentation under optimal conditions (pH 5.2 at 35°C). In comparison to parallel fermentations without preincubation (Figure 14), this preincubation step neither increased the rate nor yield of ethanol. Base consumption was slightly higher in the pretreated fermentation (52 mmoles KOH $liter^{-1}$) than for the SSF experiment (40 mmoles $liter^{-1}$), while the reverse was true for ethanol yield (pretreatment=71% and SSF=74% of theoretical yield).

Discussion

Based on comparisons of reported enzymatic conversions of cellulose to ethanol, the SSF process using recombinant *K. oxytoca* strain P2 appears to offer many advantages in performance. Wyman et al. (1978) and Spindler et al. (1992) have reported some of the most effective yeast-based conversions of cellulose to ethanol using Sigmacell 50 as a substrate and Genencor cellulase (product 150 L, no longer commercially available). These studies with the same substrate and a similar enzyme preparation allow direct comparisons. Table 3 summarizes the best results from both prior studies and includes several fermentations from the current work. To facilitate comparisons, *K. oxytoca* strain P2 data was recalculated without correction for base dilution since it is unclear whether this correction was made in prior studies. Thus the values for *K. oxytoca* strain P2 (Table 3)

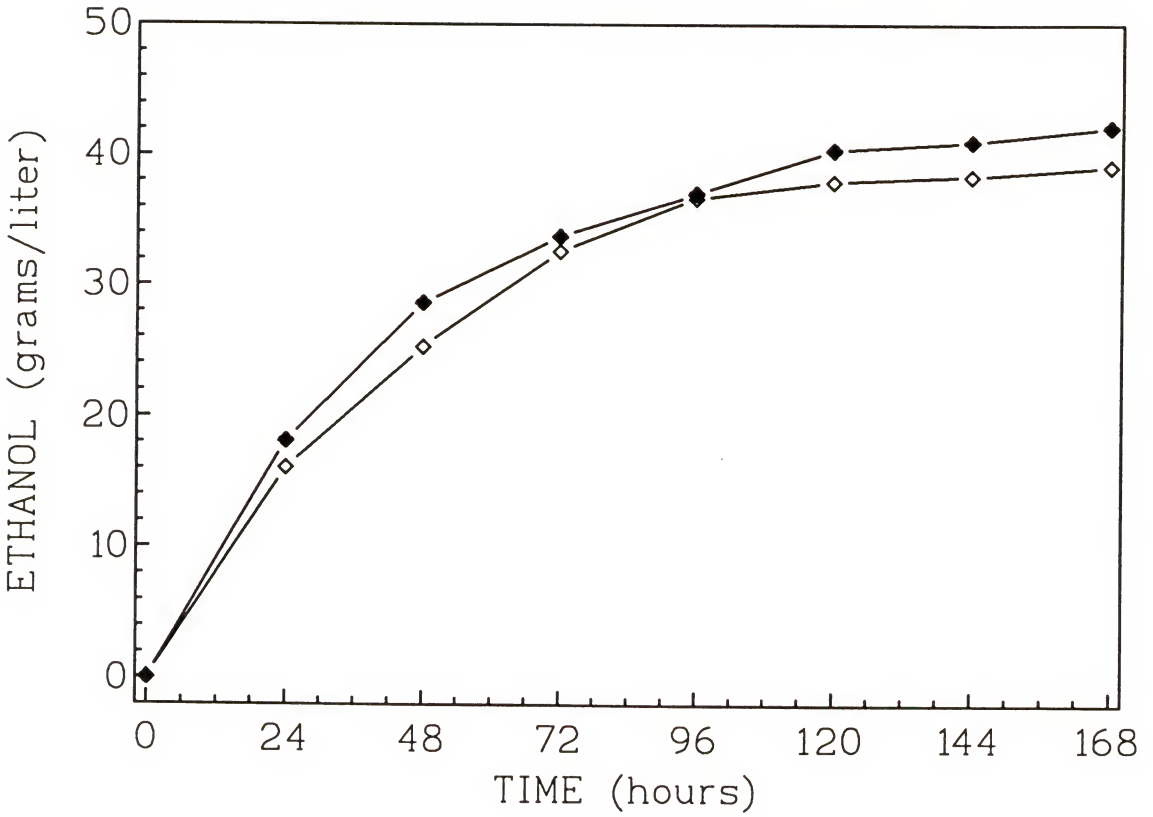


Figure 14. Effect of initial 12-h cellulose hydrolysis under optimal conditions for cellulase (pH 5.0 and 50°C; 100 g Sigmacell 50 cellulose, 10 FPU cellulase g^{-1} cellulose) prior to fermentation at pH 5.2 and 35°C. Symbols: ♦, no pretreatment; ◊, pretreated with cellulase for 12 h.

Table 3. Comparison of ethanol production from Sigmacell 50 cellulose and Genencor cellulases and different organisms.

Organism	Cellulose ($g\ L^{-1}$)	Cellulase ($FPU\ g^{-1}$)	Ethanol Production					Reference
			Time(h)	Max.($g\ L^{-1}$)	Yld($g\ g^{-1}$)[a]	% Theor. Yield[b]	$Time_{40}$(h)[c]	
S. cerevisiae[d]	100	13	288	43	0.37	67	163	Wyman et al., 1986
	100	26	192	47	0.41	74	120	"
	100	39	240	44	0.38	69	151	"
	150	26	240	62	0.38	71	55	"
B. clausenii[d]	100	13	168	42	0.36	66	144	Wyman et al., 1986
	100	26	168	43	0.37	67	134	"
	100	39	204	44	0.38	69	132	"
	150	26	168	48	0.29	55	98	"
both organisms[d]	100	13	216	49	0.43	77	110	Wyman et al., 1986
	100	26	216	50	0.43	78	72	"
	100	39	216	50	0.43	78	81	"
	150	26	216	66	0.40	76	41	"
B. custersii[e]	75	26	72	32	0.43	76	-	Spindler et al., 1992
	100	19	240	40	0.40	71	240	"
K. oxytoca P2[f]	100	10	168	42	0.42	74	117	This work
	100	20	168	45	0.45	79	96	"
	100	25	144	47	0.47	83	87	"
	140	7.2	120	45	0.32	57	78	"
K. oxytoca P2[g]	100	25	96	43	0.43	76	72	This work

[a] Grams of ethanol per gram of substrate. To allow comparison to the literature, our values have not been corrected for dilution by the addition of base during fermentation. Note that fermentations with organisms referenced to footnote [d] also contain 10 g glucose L^{-1} and 5 g cellobiose L^{-1} in addition to cellulose.

[b] Percentage of the maximum theoretical yield assuming the production of ethanol from cellulose, cellobiose, and glucose are 0.56, 0.54, and 0.51 g ethanol $gram^{-1}$ substrate, respectively. Note that fermentations with organisms referenced to footnote [d] also contain 10 g glucose L^{-1} and 5 g cellobiose L^{-1} in addition to cellulose.

[c] Time required for the fermentation to reach an ethanol concentration of 40 g L^{-1}.

[d] Medium contained 10 g glucose L^{-1}, 5 g cellobiose L^{-1}, 10 g yeast extract L^{-1}, 20 g peptone L^{-1}, 5 mg ergosterol L^{-1} and 30 mg oleic acid L^{-1}. Fermentations were conducted at 37°C. The production of ethanol from added cellobiose and glucose were included with cellulose in the calculation of substrate yield and conversion (% theoretical maximum).

[e] Medium contained 10 g yeast extract L^{-1}, 20 g peptone L^{-1}, 5 mg ergosterol L^{-1} and 30 mg oleic acid L^{-1}.

[f] Medium contained 5 g yeast extract L^{-1}, 10 g tryptone L^{-1} and 5 g NaCl L^{-1}. Fermentations were conducted at 35°C and pH 5.2.

[g] Same as [f] except fermented at 37°C, pH 5.0.

underestimate actual ethanol yields which are shown in Table 2. The highest yields and rates which were obtained by Wyman et al. (1978) used a combination of *Saccharomyces cerivisiae* and *Brettanomyces clausenii*. However, fermentable substates in this study included 100 g cellulose liter^{-1}, 10 g glucose liter^{-1} and 5 g cellobiose liter^{-1}. Cellobiose and glucose should be converted to ethanol much more rapidly than the primary substrate cellulose with a theoretical yield of 7.8 g ethanol liter^{-1} even without the breakdown of cellulose. In subsequent studies, *Brettanomyces custersii* was reported to be superior to either of the above organisms alone during the fermentation of cellulose in the absence of added sugars and represents one of the best SSF fermentations in the literature. *B. custersii* produced 32 g ethanol liter^{-1} (76% of theoretical yield) in 72 hours from 75 g cellulose liter^{-1} and 26 FPU of cellulase g^{-1} cellulose. With higher levels of cellulose (100 g liter^{-1}) and slightly lower amounts of cellulase (19 FPU g^{-1} cellulose), yields were reduced to 71% of theoretical (40 g liter^{-1}) and required 240 h for completion.

Analogous fermentations have now been conducted with *K. oxytoca* strain P2 (100 g cellulose liter^{-1} and 25 FPU g^{-1} cellulose) and produced 45-47 g ethanol liter^{-1} (81-86% of theoretical yield) after 168 h. At 37°C and pH 5.0, over 40 g ethanol liter^{-1} was produced in less than 72 h. Since cellulase enzymes are an expensive component of the SSF

process, it was encouraging to note that 42 g ethanol liter^{-1} (74% of theoretical yield) was produced with approximately half the enzyme level using *K. oxytoca* strain P2 after 168 h. Alternatively, the rate of ethanol production can be increased by a second addition of cellulose substrate without further enzyme supplements, producing 40 g ethanol liter^{-1} after 78 h with an effective enzyme loading of 7.2 FPU g^{-1} cellulose (140 g cellulose liter^{-1}). Increasing the amount of cellulose increased the rate of ethanol production and reduced the time required to reach 40 g ethanol liter^{-1}, regarded by some as a key economic breakpoint to mimimize the costs of purification (Jeffries, 1988). However, little additional ethanol was produced by *K. oxytoca* strain P2 with 140 g cellulose liter^{-1} as compared to 100 g cellulose liter^{-1} indicating that 45 to 47 g ethanol liter^{-1} may approach the upper limit for ethanol tolerance in this organism.

Increased ethanol tolerance may not be needed for commercial ethanol production from lignocellulose due to the difficulty in obtaining and mixing suspensions containing high concentrations of solids. If a goal of 85% of theoretical efficiency is set for the saccharification and fermentation processes, these can be readily achieved with *K. oxytoca* strain P2 in SSF over a wide range of conditions. This goal (approximately 41 g ethanol liter^{-1} from 100 g cellulose liter^{-1}; 72% overall efficiency) was achieved under many conditions in less than 96 h.

CHAPTER III
SACCHARIFICATION AND FERMENTATION OF SUGAR CANE BAGASSE BY *KLEBSIELLA OXYTOCA* STRAIN P2

Introduction

The conversion of wood or agricultural residues to ethanol is an attractive option for utilizing all major components of biomass to produce a liquid automotive fuel and for environmental remediation (Saddler,1993; Lynd et al., 1991). Plant biomass consists of a mixture of carbohydrate and phenolic components. Residues such as sugar cane bagasse contain 20-30% hemicellulose, 30-50% cellulose, and 10-30% lignin (Mead and Chen, 1975; Puls, 1993). Potentially, both hemicellulose and cellulose can be solubilized and fermented to ethanol.

Some form of pretreatment appears essential to render the cellulose accessible to enzymatic digestion (Saddler, 1993; Millet et al., 1976). In this regard, dilute acid hydrolysis is particularly attractive because it concomitantly hydrolyzes hemicellulose and increases the digestability of the cellulosic residue (Grohmann and Himmel, 1991). Pentose and hexose sugars generated from hemicellulose in this fashion have been efficiently fermented to ethanol by genetically engineered *Escherichia coli* which harbor genes encoding

ethanol-producing enzymes from *Z. mobilis* (Barbosa et al., 1992; Hahn-Hagerdal et al., 1993; Beall et al., 1992; Lawford and Rousseau, 1991a; Lawford and Rousseau, 1991b). In an optimal system, cellulose can also be converted into ethanol by a separate, simultaneous saccharification and fermentation (SSF) process similar to that originally developed by the Gulf Oil Company (Emert et al., 1983). Burning unsaccharified carbohydrate plus lignin could provide sufficient energy for ethanol recovery (Kerstetter and Lyons, 1991).

The cost-effective saccharification of cellulose is the most significant remaining problem for a biomass to ethanol process. The traditional SSF process with *Saccharomyces* offered an advantage by fermentatively removing glucose and thus minimizing cellobiase inhibition (Grohmann, 1993; Holtzapple et al., 1991; Gauss et al., 1976). This process was significantly improved by using other yeasts (Spindler et al., 1992) and genetically engineered *Klebsiella oxytoca* (strain P2) capable of transporting and metabolizing cellobiose (Doran and Ingram, 1993). Continuous removal of soluble products by strain P2 during the enzymatic hydrolysis of cellulose promotes saccharification by preventing the accumulation of glucose, cellobiose, and cellotriose.

Previous studies of SSF fermentations using Sigmacell 50 (highly crystalline cellulose) as a model substrate have defined the range of optimal conditions for ethanol production for strain P2 (Chapter II; Doran and Ingram, 1993). Using

this model substrate, broad optima for temperature and pH were observed which allowed the production of over 44 g ethanol liter^{-1} (82-87% of the maximum theoretical yield).

In this study, we have extended our investigations of the SSF process with *K. oxytoca* strain P2 (Wood and Ingram, 1992) by using a natural substrate, sugar cane bagasse.

Materials and Methods

Bacterial Strain

K. oxytoca strain P2 has been previously described (Wood and Ingram, 1992) and was maintained on Luria broth (containing per liter: 20 g of glucose, 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 40 mg of chloramphenicol) or medium solidified with 1.5% agar.

Preparation of Inocula for Fermentations

Cells were inoculated from a single colony to 500-ml flasks containing 200 ml of Luria broth with 50 g glucose liter^{-1}. Cultures were incubated for 24 hours at 30°C without agitation. Cells were harvested by centrifugation and used to inoculate SSF fermentations at an initial cell density of 330 mg dry weight liter^{-1}.

Fermentation Experiments

Fermentations were conducted in modified 500-ml fleakers containing 350 ml of broth as previously described (Beall et al., 1991). Glucose was replaced by varying concentrations of acid hydrolysed, ammonia freeze exploded (AFEX) (Holtzapple et al., 1991) or untreated sugar cane bagasse. Complex nutrients

(2X) were sterilized by autoclaving at 121°C for 15 minutes. Sugar cane bagasse was autoclaved at 121°C for 30 minutes. Concentrated hydrochloric acid was used to adjust the intial pH as needed. A 2 M solution of KOH was used to maintain pH during fermentation. All fermentations were conducted at pH 5.2 and 35°C. Enzyme preincubations varied as described.

Spezyme CE cellulase was provided by Genencor International (South San Francisco, CA) and contained 103 filter paper units (FPU) ml^{-1}. This enzyme preparation was filter sterilized prior to use and added at a final concentration of 10 or 20 FPU g^{-1} bagasse dry weight at the time of inoculation, unless otherwise specified.

Bagasse Pretreatments

Florida sugar cane bagasse (Sugar Cane Growers Cooperative of Florida) was hydrolyzed in dilute sulfuric acid by BioEnergy International (Gainesville, Florida) in a rotating reactor with direct steam injection (Beall et al., 1992). The residue was collected by centrifugation and washed with distilled water (filtration) until pH 5.0 was achieved. Excess moisture was removed by centrifugation and the lignocellulose residue stored at -20°C. Where indicated, particle size was further reduced by grinding in a Krups coffee mill. Although acid-treated bagasse consisted of a mixture of particle sizes, cane fragments of 1-3 cm in length represented over half of the dry weight. After grinding, particle sizes were reduced to approximately 1-2 mm.

AFEX-treated sugar cane bagasse was generously provided by Dr. Bruce Dale (Holtzapple et al., 1991). The particle size of this material was similar to that of acid-treated bagasse which had been ground.

Analyses

Samples were removed for the measurement of ethanol (g $liter^{-1}$) by gas-liquid chromatography (Beall et al., 1991). Ethanol yields were corrected for dilution by the addition of base during fermentations and computed on the basis of total carbohydrate initially present. No corrections were made for unused carbohydrate or for the production of cell mass. The maximum theoretical yield was assumed to be 0.568 g ethanol g^{-1} cellulose. Maximum volumetric productivity was estimated from the intial 24-hour period. All results represent averages from two or more fermentations, except where indicated.

The analyses of bagasse composition were based on the sequential gravimetric procedures described by Allen et al.(1975). Carbohydrates were analyzed with a Millipore/Waters high-performance liquid chromatograph (Millipore Corp., Bedford Mass.) equipped with a refractive index monitor and an electronic integrator. Separations were performed using an Aminex HPX-87P column (Bio-Rad Laboratories, Richmond, Calif.) at 85°C. AFEX treated-bagasse was determined to contain 59% carbohydrate; acid-treated bagasse residue contained 60.8% carbohydrates. The composition (% dry weight) of the fresh bagasse samples prior to acid treatment by BioEnergy Intl. was

approximately 42.4% cellulose, 27.5% hemicellulose sugar, 3.0% hemicellulose derived acetate, 27.1% lignin + other components.

Electron Microscopy

SSF samples were fixed for 4 h at room temperature in 0.1 M cacodylate buffer containing 2% glutaraldehyde and 2% formaldehyde. After washing in 0.1 M cacodylate buffer, samples were incubated for an additional 4 h in 0.1 M cacodylate buffer containing 1% osmium tetroxide. Samples were washed in distilled water, placed into 1% uranyl acetate for 2 h, rinsed in distilled water, dehydrated in ethanol and hexamethyldisilazane (Bozzola and Russell, 1992). Dry specimens were mounted on stubs, sputter coated with gold, and photographed using a Hitachi S4000 Scanning Electron Microscope (SEM).

Results

Summary of Fermentations

Table 4 summarizes the results from a large number of SSF experiments utilizing bagasse as a substrate. Representative fermentations are shown in Figure 15. Although all studies were conducted for 168 h, the time required to reach the maximum concentration of ethanol varied between 120 and 144 h for most experiments.

Effects of Pretreatment

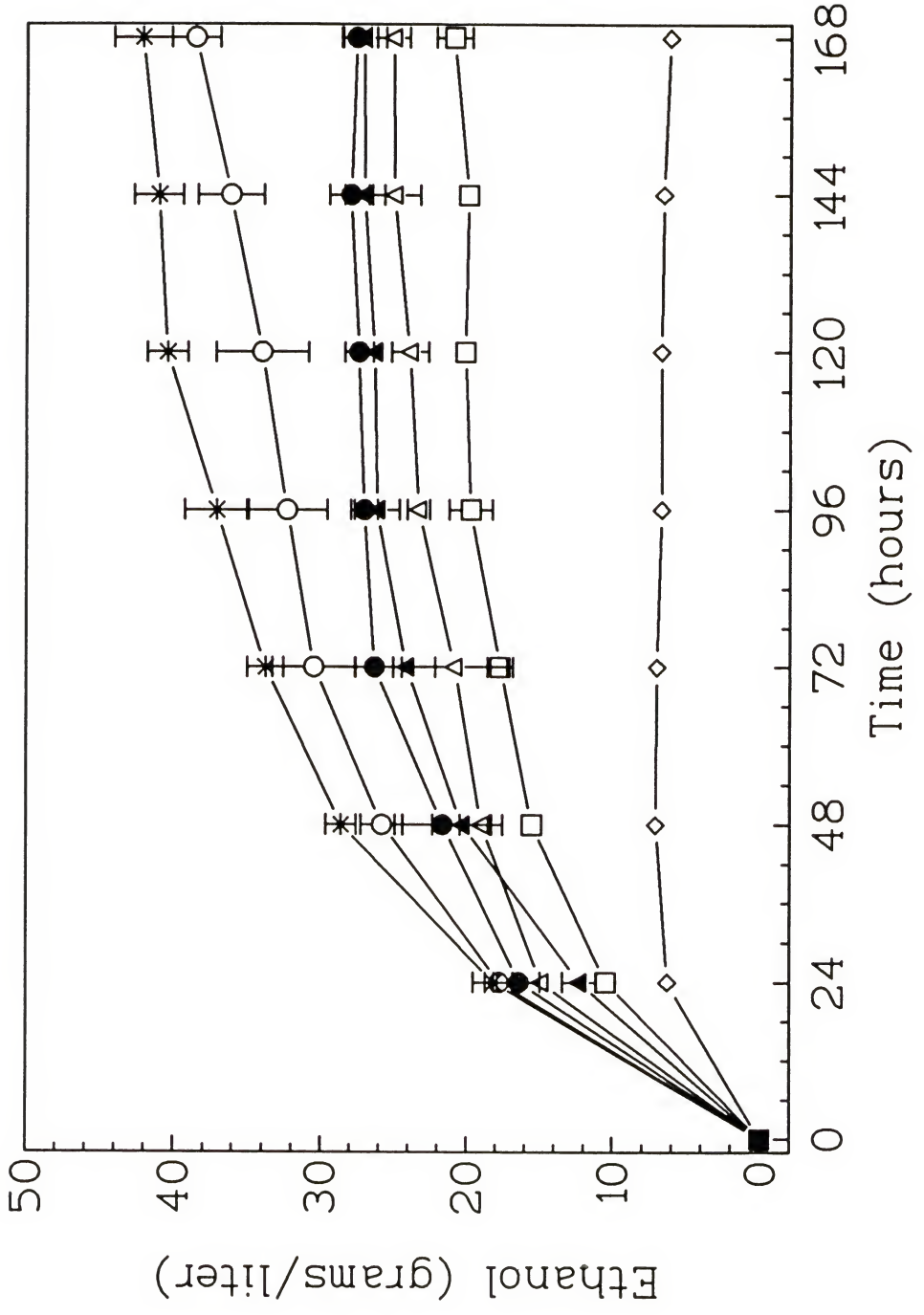
Ground (or unground) bagasse (100 g liter^{-1}) was a poor substrate for SSF unless pretreated with either ammonia

Table 4. Fermentation Summary

Pretreatment	Replicates	Bagasse[a] Concentration (g L^{-1})	G[b] (+/-)	Enzyme Conc. (FPU g^{-1})	Enzyme Preinc.[c] (hours)	Maximum Ethanol (g L^{-1})	T to Max[d] Ethanol (hours)	Base Consumed (mmoles L^{-1})	Yield (g Ethanol g^{-1} Bagasse)	Volumetric[e] Productivity (g/L/h)
Acid	2	50	-	10	0	12.5	120	42.9	0.26	0.40
Acid	2	50	-	20	0	19.2	144	32.9	0.39	0.56
Acid	2	60	-	10	0	17.0	120	28.6	0.30	0.34
Acid	5	100	-	10	0	24.2	144	22.6	0.25	0.43
Acid	2	100	+	10	0	25.1	144	8.6	0.25	0.62
Acid	2	160	-	10	0	23.6	120	10.0	0.16	0.54
Acid	1	160	-	10	24	26.4	168	10.0	0.17	0.62
Acid	1	160	+	10	12	27.7	168	10.0	0.17	0.46
Acid	1	160	+	10	24	28.2	144	17.1	0.18	0.54
Acid	2	100 + 60	+	10	24	23.9	96	27.1	0.15	0.56
Acid	2	100 + 60	+	20	24	33.8	120	20.5	0.21	0.67
Acid	2	160	-	20	24	28.0	168	17.1	0.18	0.68
Acid	5	160	+	20	24	38.6	168	17.7	0.24	0.74
Acid	2	160	+	10	12	27.7,26.7	168,72	21.4,21.4	0.17,0.17	0.46, 0
Acid	2	160	+	10	12	27.7,39.2	168,72	21.4,25.7	0.17,0.25	0.46,0.20
None	2	100	+	10	24	7.1	48	2.9	0.07	0.26
Sigmacell[f]	5	100	+	10	0	42.1	168	40.0	0.43[f]	0.76
AFEX	4	100	+	10	0	20.5	144	20.7	0.21	0.44

[a] Dry weight.
[b] Ground (+ yes, - no)
[c] Preincubation at 45°C, pH 4.5-4.8
[d] Time required to reach maximum ethanol concentration.
[e] Grams of ethanol produced per liter per hour for the first 24 hours of fermentation.
[f] Sigmacell 50 crystalline cellulose.

Figure 15. Representative SSF experiments using sugar cane bagasse or Sigmacell 50 cellulose as substrates with 10 FPU Spezyme CE cellulase g^{-1} substrate (except where indicated). Symbols are defined as follows: 100 g $liter^{-1}$ Sigmacell 50 cellulose (*); 100 g $liter^{-1}$ ground, untreated bagasse (◊); 100 g $liter^{-1}$ AFEX-treated bagasse (□), 100 g $liter^{-1}$ ground, acid-treated bagasse (△), 160 g $liter^{-1}$ ground, acid-treated bagasse which had been preincubated under optimum saccharification conditions (pH 4.8, 48°C) for 24 h prior to inoculation (▲), 160 g $liter^{-1}$ acid-treated bagasse which had been preincubated for 24 h with 20 FPU cellulase g^{-1} bagasse prior to inoculation [unground (●), ground (○)].


Ethanol (grams/liter)
50
40
30
20
10
0
0
24
48
72
96
120
144
168
Time (hours)

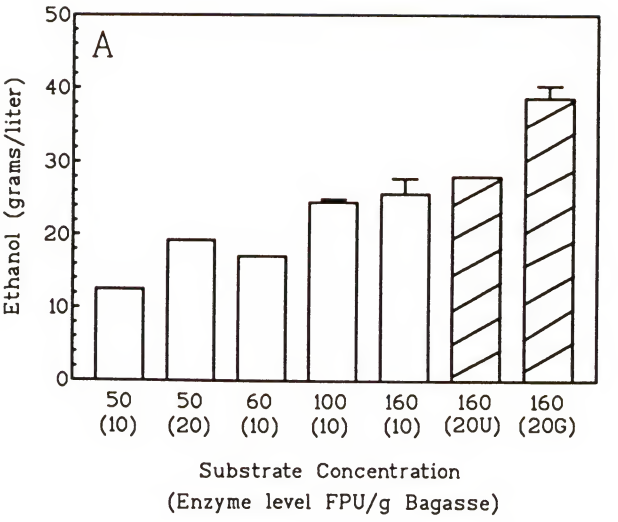
explosion (AFEX) or dilute acid hemicellulose hydrolysis. Using 10 FPU cellulase g^{-1} bagasse and a 24 h preincubation with enzyme prior to inoculation, only 7 g ethanol $liter^{-1}$ was obtained after 168 h. Both AFEX and acid hydrolysis of hemicellulose increased cellulose digestability by 3-fold. SSF of AFEX-treated and acid-treated bagasse (10 FPU cellulase g^{-1} bagasse) produced approximately 21 g ethanol $liter^{-1}$ and 25 g ethanol $liter^{-1}$, respectively, after 168 h and achieved over 70% of the theoretical yield. Although acid treatment was somewhat better than the single AFEX condition examined, it is likely that both treatments could be equally effective for bagasse under optimized conditions. Further studies were conducted using only the acid-treated bagasse.

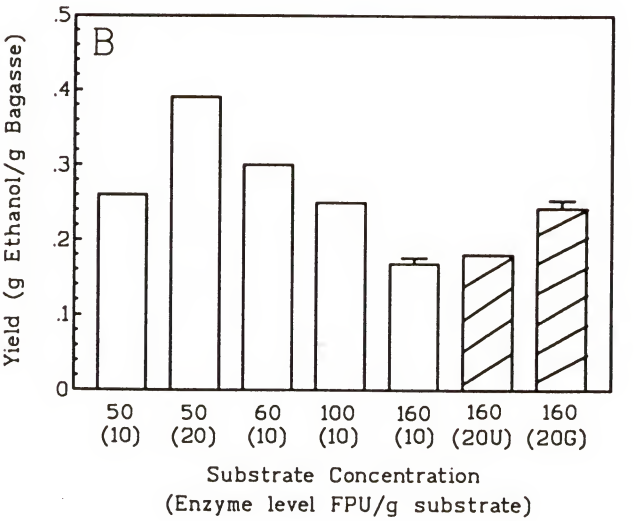
Effects of Bagasse Concentration

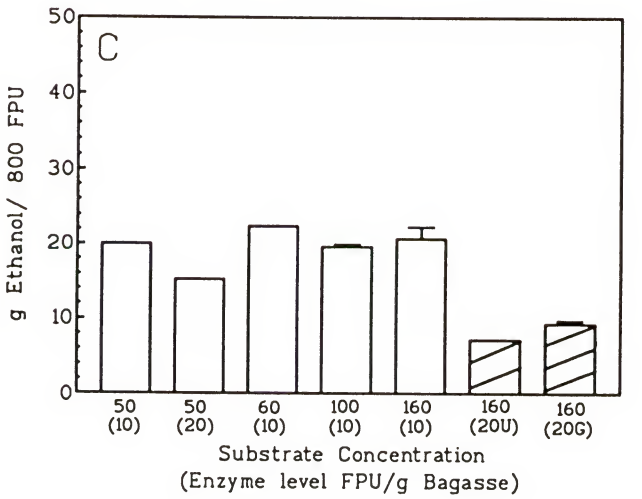
We have examined the effects of different bagasse concentrations on ethanol yields at two levels of cellulase, 10 FPU g^{-1} bagasse and 20 FPU g^{-1} acid-treated bagasse (Figure 16, Table 4). Initial rates of fermentation and maximal ethanol concentrations increased in response to higher levels of acid-treated bagasse and to higher levels of cellulase, confirming that SSF is limited by saccharification (Figure 16A).

Ethanol yields $gram^{-1}$ of acid-treated bagasse were highest at low concentration of acid-treated bagasse, 50 to 60 g $liter^{-1}$ (Figure 16B). A doubling of cellulase increased ethanol yield.

Figure 16. Effect of substrate concentration (g liter^{-1}) and enzyme level on ethanol production. (A) Maximum ethanol production. (B) Ethanol yield. (C) Ethanol produced per 800 FPU cellulase. Shaded bars represent a comparison of particle sizes using 160 g bagasse liter^{-1} and 20 FPU enzyme g^{-1} bagasse (U, unground; G, ground).


A
Ethanol (grams/liter)
0
10
20
30
40
50
50 (10)
50 (20)
60 (10)
100 (10)
160 (10)
160 (20U)
160 (20G)
Substrate Concentration
(Enzyme level FPU/g Bagasse)


B
Yield (g Ethanol/g Bagasse)
0
.1
.2
.3
.4
.5
50 (10)
50 (20)
60 (10)
100 (10)
160 (10)
160 (20U)
160 (20G)
Substrate Concentration
(Enzyme level FPU/g substrate)


C
g Ethanol/ 800 FPU
0
10
20
30
40
50
50 (10)
50 (20)
60 (10)
100 (10)
160 (10)
160 (20U)
160 (20G)
Substrate Concentration
(Enzyme level FPU/g Bagasse)

Ethanol production had essentially ended after 168 h with 100 g^{-1} liter bagasse and 10 FPU cellulase g^{-1} bagasse although little more than 70% carbohydrate had been metabolized. This cessation of ethanol production was not due simply to a lack of viable biocatalyst since a second inoculation with strain P2 at this time did not lead to further ethanol production. To determine if the SSF was limited by cellulase activity, a second addition of enzymes (10 FPU g^{-1} original bagasse) was made but little increase in ethanol occurred (1 g ethanol $liter^{-1}$ after an additional 96 h). In contrast, the addition of 40 g Sigmacell 50 $liter^{-1}$ at the end of 168 h SSF resulted in an increase in ethanol concentration from 25 g $liter^{-1}$ to 30 g $liter^{-1}$. These results indicate that the enzymes remain catalytically active. A portion of the bagasse cellulose appears to be recalcitrant and not readily degraded. Based on yields obtained with 100 g bagasse $liter^{-1}$ and 10 FPU g^{-1} bagasse, the recalcitrant fraction appears to represent 25% of the total carbohydrate in acid-treated bagasse. For comparison, a highly purified model cellulose (Sigmacell 50, 10 FPU cellulase g^{-1} fermentable carbohydrate) was also examined. SSF with this substrate (100 g $liter^{-1}$) produced 42 g ethanol $liter^{-1}$ after 117 h, with an equivalent yield (74% of theoretical yield) and similar recalcitrant fraction.

The cost of cellulases is a crucial factor in the economics of a bioconversion process. Thus it is also instructive to compare the ethanol produced as a function of

total enzyme utilized (Figure 16C). The value, 800 FPU, was selected because it represents the approximate activity gram^{-1} of commercial cellulase protein (fungal). From this figure, it is apparent that lower substrate concentrations (below 100 g bagasse liter^{-1}) with the lower enzyme level (10 FPU g^{-1} bagasse) provide most efficient enzyme utilization. Higher concentrations of substrate or higher enzyme loadings were much less effective. By using a second saccharification step, 20 g of ethanol per gram of fungal protein was obtained compared to 32 g ethanol g^{-1} fungal protein using Sigmacell 50 cellulose as the substrate.

Viscosity Reduction Prior to SSF

The decrease in enzyme effectiveness at the highest level of bagasse may be due in part to high viscosity and reduced mixing. Since a bagasse concentration of 160 g liter^{-1} is needed to produce over 40 g ethanol liter^{-1} at 70% of theoretical yield, three approaches were investigated to increase the effectiveness of cellulase enzymes under high substrate conditions: 1) sequential addition of bagasse during the SSF fermentation; 2) grinding to reduce particle size; 3) and preincubation under conditions optimal for cellulase prior to inoculation (Table 4). Batch feeding of acid-treated bagasse during the initial 24 h of SSF did not improve fermentation yields using a variety of regimens. Reducing the particle size had no significant effect on

ethanol yield with 10 FPU cellulase g^{-1} bagasse but did significantly improve our mixing and pH control.

Preincubation with cellulase (10 FPU cellulase g^{-1} acid-treated bagasse, 160 g bagasse $liter^{-1}$, 45°C, pH 4.5) prior to inoculation also reduced viscosity and increased mixing. However, preincubation alone resulted in only a small increase in ethanol yield. A further modest increase was observed with a combination of grinding and pretreatment. In this regard, a 12-h preincubation was essentially equivalent to a 24-h pretreatment. To obtain ethanol levels which approach 70% of theoretical yield, twice the level of cellulase was needed (20 FPU g^{-1} acid-treated bagasse) and produced 38.6 g ethanol $liter^{-1}$ (Figure 16). With this combination approach, however, ethanol production 800 FPU^{-1} enzyme (9.6 g) remained far below that achieved with more dilute substrate and half the enzyme loading (20-22 g).

Ultrastructural Investigations

SSF samples were removed and examined after various stages of fermentation. After 12 h to 24 h of enzyme treatment, much of the ragged structure was removed from the surface of Sigmacell 50 (Figure 17A,B) and bagasse samples, consistent with previous reports (Din et al., 1991). Cell adherence was also investigated during SSF (Figure 17C,D,E). As illustrated by samples removed after 168 h of SSF, few P2 cells adhered to the highly purified substrate, Sigmacell 50. Many areas were observed in which cells were absent.

Figure 17. SEM photographs of fermentation samples. (A) Sigmacell 50 cellulose prior to fermentation. (B)Sigmacell 50 cellulose after 6 h SSF. Figures C, D, and E are SEM photographs of fermentation samples removed after 168 h SSF: (C) Acid-treated bagasse. (D) AFEX-treated bagasse. (E) Sigmacell 50 cellulose.

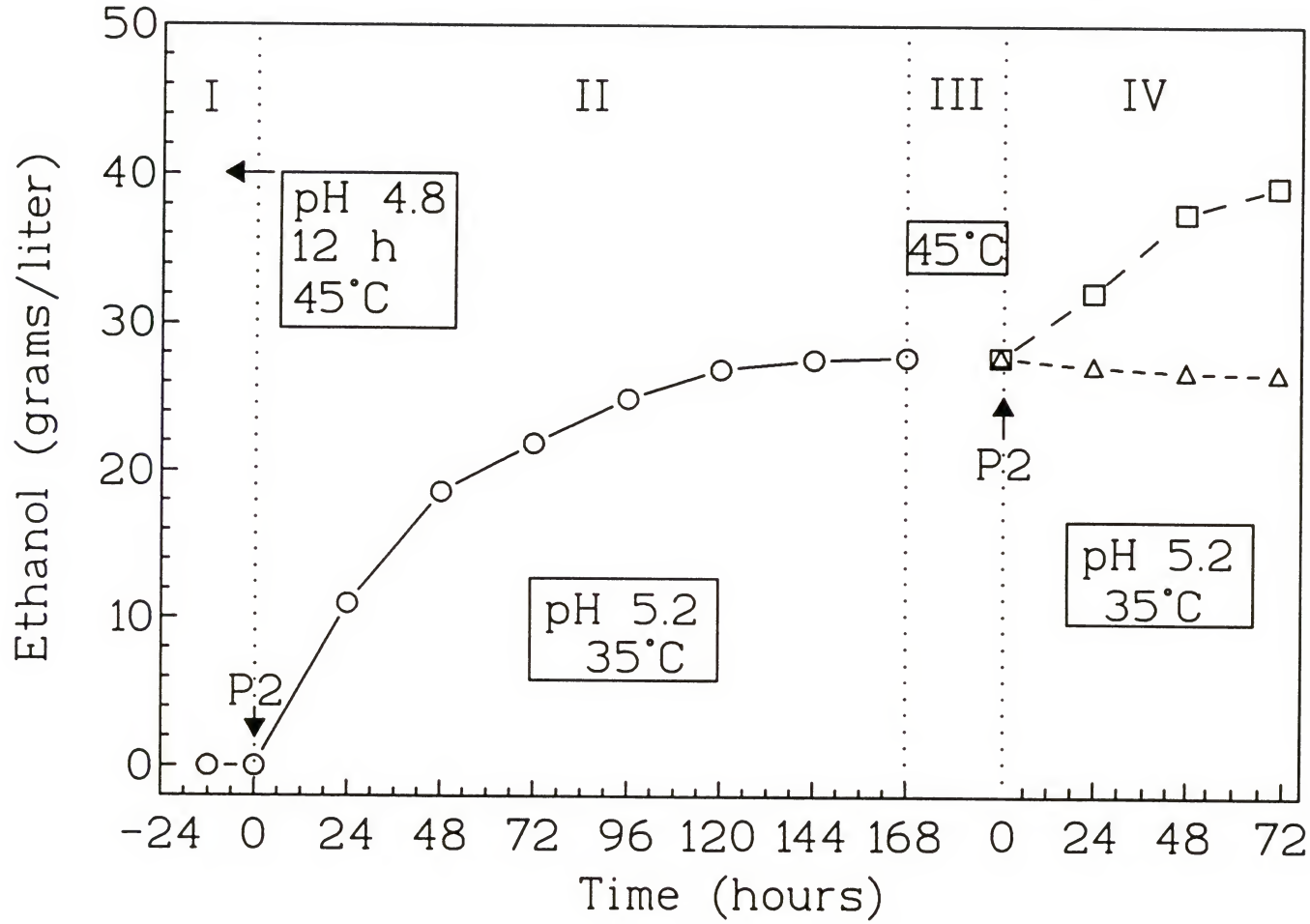

A
10μm
B
10μm
C
10μm
D
10μm
E
10μm

Adherence to AFEX-treated bagasse was most extensive; adherence to acid-treated bagasse was intermediate. It is tempting to speculate that this trend of adherence may be related to differences in hemicellulose composition. Sigmacell 50 contains essentially no hemicellulose; most hemicellulose is hydrolyzed and removed by dilute acid pretreatment. However, with the AFEX treatment, hemicellulose components are redeposited on the surface and appear to represent an excellent matrix for strain P2 adherence (Figure 17D).

Improvement of SSF by a Second Saccharification Step and Re-inoculation

Although cellulase remained active in SSF experiments after 168 h, almost half of the cellulose was not converted to ethanol using 160 g bagasse liter^{-1} and 10 FPU cellulase g^{-1} bagasse. Further studies were conducted to improve the effectiveness of enzyme utilization. After 168 h, the SSF was shifted to 45°C to favor saccharification. Following a 24-h incubation, the suspension was cooled, adjusted to pH 5.2, and reinoculated with strain P2 (Figure 18). A doubly inoculated culture which was not shifted in temperature served as a control. After 72 h, a significant increase in ethanol was obtained, from 27.7 g liter^{-1} to 39.2 g liter^{-1}. The ethanol produced after this two-step fermentation was equivalent to 73% of the theoretical yield (correcting for dilution by base) and increased the effective yield of ethanol per 800 FPU cellulase by over 40% (20 g ethanol 800 FPU^{-1} cellulase).

Figure 18. Improvements in SSF by the use of a second saccharification step and reinoculation. An enzyme preincubation step was conducted for 12 h at pH 4.8 and 45°C prior to inoculation (stage I); followed by SSF (○), pH 5.2 and 35oC for 168h (stage II). A second saccharification step at 45°C, pH 5.0-4.8, was conducted for 24h (stage III), then cooled to 35°C and the pH adjusted to 5.2 (stage IV). Strain P2 was added to cultures which had been subjected to a second saccharification step (□) and to cultures which were kept at 35°C (△). Addition of strain P2 is indicated by arrows.


Ethanol (grams/liter)
50
40
30
20
10
0
I
II
III
IV
pH 4.8
12 h
45°C
45°C
P2
P2
pH 5.2
35°C
pH 5.2
35°C
-24
0
24
48
72
96
120
144
168
0
24
48
72
Time (hours)

Discussion

The use of acid hydrolysed sugar cane bagasse as a substrate with recombinant *K. oxytoca* strain P2 as the biocatalyst has excellent potential for fuel ethanol production. From each dry metric ton of this lignocellulosic material, approximately 300 kg of sugar are produced by acid hydrolysis which can then be fermented to produce 170 liters of ethanol (90% efficiency) by recombinant *E. coli* strain KO11 (Ingram, unpublished). The cellulose residue can be readily digested by commercial cellulases in an SSF process using *K. oxytoca* strain P2. In the optimal process described, almost 40 g ethanol liter^{-1} were produced with 10 FPU cellulase g^{-1} acid-treated bagasse, over 70% of the theoretical yield. Thus the cellulosic portion of each metric ton of acid-treated cellulose will yield an additional 185 liters of ethanol. By combining these yields, the projected yield of ethanol (355 liters metric ton^{-1}) is similar to that obtained per metric ton of corn in current U.S. processes. Remaining lignin and carbohydrate residues from a bagasse-based process could provide a major portion of the energy needed for the distillation.

The second saccharification step was surprisingly effective and could result from a combination of several actions. The increase in ethanol production after a temperature shift may be due to to enhanced nicking of cellulose chains to produce new nonreducing termini for

digestion. One explanation proposed previously for the progessive decline in cellulose hydrolysis is that the free cellulose ends, generated by initial pretreatments, are consumed faster than they can be regenerated (Converse, 1993). Woodward et al. (1988) reported that the initial supply of free chain ends was rapidly depleted during saccharification, and that cellobiohydrolase action becomes dependent upon the rate at which new ends are generated by endoglucanases. Further support for this hypothesis is provided by ultrastructural investigations which demonstrated the speed with which ragged edges of cellulose particles (ramie fiber) were digested to leave the more recalcitrant smooth cellulose fibrils (Din et al., 1991). The second elevated temperature treatment could also help in other ways. For instance, elevated temperatures could facilitate the release of cellulase enzymes from nonproductive binding sites with subsequent attachment to new regions where the cellulose chains are more digestable.

CHAPTER IV
ISOLATION AND CHARACTERIZATION OF NATURALLY CELLULOLYTIC MICROORGANISMS BIOCHEMICALLY SIMILAR TO *KLEBSIELLA*

Introduction

Klebsiella oxytoca is a soil organism that is abundant in cellulosic waste streams from the pulp and paper industry (Grimont et al., 1991). This organism has been shown to efficiently metabolize xylo-oligosaccharides (Burchhardt et al., 1992) and gluco-oligosaccharides (Al-Zaag, 1989; Wood and Ingram, 1992). Although no truly cellulolytic *Klebsiella* is currently available, *Klebsiella* sp. appear to inhabit areas rich with cellulosic residues. Typically, in the soil environment researchers culture only 1 % of the organisms observed by light microscopy (Tiedje, 1994). This ecosystem probably contains cellulolytic *Klebsiella* which could prove useful in an industrial process. *K. oxytoca* is amenable to metabolic engineering by integrating the genes for ethanol production from *Zymomonas mobilis* into the chromosome (Ohta et al., 1991ab). This strain, designated *K. oxytoca* P2, has been useful in simultaneous saccharification and fermentation experiments using Sigmacell 50 (a model crystalline cellulose) (Chapter II; Doran and Ingram, 1993) and sugar cane bagasse (Chapter III; Doran and Ingram, 1994). Although the P2 strain

utilizes cellobiose, it lacks the other components necessary to degrade cellulose, namely the endo- and exo-glucanase activities. In current processes, the cellulase activities are supplied by the addition of a commercially available fungal preparation. Genes encoding cellulase activity could be cloned from the naturally cellulolytic organism and expressed in *K. oxytoca* strain P2. Although there have been cellulase genes cloned from many bacterial species, genes from an organism of the same species (or from an organism that is very similar genetically) may be more efficiently processed.

Materials and Methods

Isolation Procedure

Samples were removed from nine sites at a cellulose processing plant in Perry, Florida. All sites sampled were at pH 8.0 and between 28°C and 31°C. Serial dilutions were plated onto *Klebsiella* medium (Atlas, 1993) containing the following ingredients per liter 5.0 mg carbenicillin; 0.04 g sodium taurocholate; 0.1 g Phenol Red; 0.3 g Uric acid; 4.0 g adonitol; 8.0 g KCL; 3 g K_2HPO_4; 2 g NaCl; 1.0 g KH_2PO_4; 0.2 g $MgSO_4$-$7H_2O$; and 15.0 g agar. Plates were incubated at 30°C and examined for growth at 24 h and 48 h.

Colonies were picked to a modified Luria agar containing the following ingredients per liter 10.0 g tryptone; 5.0 g yeast extract; 5.0 g sodium chloride; 0.04g sodium taurocholate; 5.0 mg carbenicillin; 2.0 g carboxymethyl-cellulose (CMC) (low viscosity, sodium salt); and 15.0 g agar.

Screening for Carboxymethylcellulase Activity (CMCase)

Colonies were screened for CMCase activity by replica plating onto modified Luria agar described above. After overnight incubation at 30°C, cells were washed from the surface of the medium and CMC-hydrolysis positive colonies were identified using the Congo Red staining procedure (Teather et al., 1982). After single colony isolation, CMCase positive microorganisms were rescreened on *Klebsiella* selective medium and modified Luria agar containing CMC.

Biochemical Assays for Classification

Biochemical assays were conducted as described in *Bergey's Manual of Systematic Bacteriology* (Brenner, 1984).

Fatty Acid Analysis

Fatty acid analysis was performed on cultures in mid-log phase of growth by Dr. Robert Stall and collegues (Stall et al., 1994). Cultures were passed through cellulosic 0.45-μm-pore-size filters, and approximately 40 mg (wet weight) of cells was transferred to glass test tubes (13 by 100mm) fitted with Teflon-lined caps for fatty acid extraction. Cellular fatty acids were extracted and derivatized to their methyl esters (FAME) according to the method of Miller (Miller, 1982). Statistical analysis of FAME profiles was conducted with MIDI library Generation System Software (Microbial ID, Inc., Newark, DE).

Bacterial Strains and Plasmids

Strain JD2A5 was used as the source of DNA for cloning. *Escherichia coli* DH5α was used as the recipient strain for transformation during library screening and was grown at 37°C in Luria broth (Sambrook et al., 1989) or Luria agar (1.5% agar). Plasmid pUC18 was used as the cloning vector. *Klebsiella oxytoca* strain P2 (Wood and Ingram, 1992) was also used as a recipient strain for transformation. The medium for recombinant *E. coli* DH5α, or *K. oxytoca* strain P2, containing cloned CMCase genes was supplemented by 50 mg of ampicillin per liter.

Construction of Plasmid Library in *E. coli*

Strain JD2A5 was grown at 30°C on Luria agar for 24 h. Colonies were replica plated to LB CMC plates and a single colony showing a zone of hydrolysis was selected as an inoculum for 50 ml of Luria broth. Cell growth was at 30°C in a shaking water bath (100 rpm) for 24 hours. Total DNA from strain JD2A5 was prepared as described by Cutting and Vander Horn (1990). Purified JD2A5 DNA was partially digested with *Sau*IIIA. The digest was separated on a 1% agarose gel and fractions from 2-4kb were used to construct a library in PUC18 as described previously (Byun et al., 1986). Clones were selected for ampicillin resistance and zones of hydrolysis on CMC plates with DH5α as the host. Approximately 2700 colonies were pooled from the original transformation plates and used to isolate plasmid DNA (Sambrook et al., 1989).

DNA Sequencing and Analysis

Double-stranded plasmid DNA was purified for sequencing by the use of Magic Mini Prep columns (Promega Corp., Madison, WI). The CMCase-clone from JD2A5 (pLOI1323) was sequenced using a LI-COR (Lincoln, NE) model 4000 DNA sequencer by the Dept. of Microbiology and Cell Science DNA Sequencing Facility (Sanger et al., 1977). Resulting sequences were analyzed with the Genetics Computer Group sequence analysis software package (Devereux et al., 1991) and the National Center for Biotechnology Information data base searches using BLAST network server (Altschul et al., 1990). Amino acid sequences were aligned using the CLUSTAL V program (Higgins et al, 1992).

Testing for Other Hydrolytic Traits

Putative cellobiohydrolase and ß-glucosidase activities were tested on Luria agar containing 10 mg of 4-methylumbelliferyl-ß-D-glucopyranoside (MUG) or 4-methylumbelliferyl-ß-D-cellobiopyranoside (MUC) per liter (Wood et al., 1988). Xylosidase, mannosidase, and arabinosidase activities were screened in a similar manner with the respective umbelliferyl derivitives. Starch hydrolyzing activity was tested on Luria agar containing 10 g soluble starch per liter. Clear zones indicating starch hydrolysis were observed by staining plates with an iodine and potassium iodide mixture (3.3 g and 6.6 g per liter, respectively).

Results

Isolation of Organisms

Samples were removed from nine sites at a Florida cellulose plant. Serial dilutions in phosphate buffered saline were plated onto *Klebsiella* selective agar and modified Luria agar containing carboxymethylcellulose and were incubated at 30°C for 48 h. Approximately 250 positive colonies were plated for isolated colonies and retested. Forty colonies with large zones of hydrolysis on CMC-agar and which grew on *Klebsiella* medium were examined for Gram stain characteristics. Of these, 25 (62%) were Gram-negative rods. The remainder were either Gram-positive rods (15%), or a mixture of Gram-negative rods with Gram-positive cocci in clusters (23%). The 25 Gram-negative rods were tested for oxidase production and for anaerobic growth with glucose as the carbon source. Three oxidase negative, facultatively anaerobic organisms were chosen for further biochemical characterization and were designated JD2A5, JD2J3, and JD2J12. The results of the biochemical characterizations are presented in Table 5.

Fatty Acid Analysis

The three *Klebsiella*-like organisms, JD2A5, JD2J3, and JD2J12, were subjected to fatty acid analysis. A fourth strain, JD9F, was an obligately aerobic Gram-negative rod that was classified as a strain of *Pseudomonas* using biochemical methods from *Bergey's Manual* (data not shown). Strain JD9F

Table 5. Biochemical Analysis of Isolated Strains and Selected Members of *Enterobacteriaceae*.[a]

	Strain					
Characteristics	JD2A5	JD2J3	JD2J12	*K. oxytoca*[b]	*K. planticola*[b]	*E.agglomerans*[b]
Motility	+	-	-	-	-	[+]
Indole Production	+	+	+	+	d(+)	[-]
Voges-Proskauer	+	+	+	+	+	d
Citrate, Simmon's	+	+	+	+	+	d
Hydrogen Sulfide on TSI	-	-	-	-	-	-
Urease, Christensen's	+	-	-	+	+	[-]
Phenylalanine Deaminase	-	+	-	-	-	[-]
Lysine Decarboxylase	-	-	-	+	+(-)	-
Arginine Dihydrolase	-	-	-	-	-	-
Ornithine Decarboxylase	-	-	-	-	-	-
Pectate Degradation	-	-	-	+	-	-
D-Glucose, Acid	+	+	+	+	+	+
D-Glucose, Gas	+	+	-	+	+	[-]
Adonitol	+	+	-	+	d(-)	-
Cellobiose	+	+	+	+	+	d

Lactose	+	+	+	+	+	d
L-Arabinose	+	+	+	+	+	+
Xylose	+	-	-	+	+	+
Sucrose	+	+	+	+	+	[+]
D-Mannitol	+	+	+	+	+	+
Dulcitol	-	-	-	d(-)	d(-)	[-]
Carbinicillin Resistant	+	+	+	+	+	d
α-Methyl-D-Glucoside	+	+	+	+	nd(+)	-
Esculin hydrolysis	+	nd	nd	+	nd	d

[a] Symbols from Bergey's Manual:

+	90-100% of strains are positive
[+]	76-89% of strains are positive
d	26-75% of strains are positive
[-]	11-25% of strains are positive
-	0-10% of strains are positive
nd	not determined

[b] Based on published results in *Bergey's Manual*. Results were confirmed for *K. oxytoca* strain M5A1 and *K. planticola* strain 8942. Where results are different is indicated in parentheses to the right.

was included in the fatty acid analysis profile for comparison. A laboratory strain of *Klebsiella oxytoca,* strain M5A1, was also included in the analysis. Based on the MIDI dendrogram software program, the population was linked with a Euclidian distance greater than 25, indicating that the JD9F strain is a separate genus from the other strains. All JD strains and strain M5A1 were linked within a Euclidian distance of 25, suggesting these strains are likely to be members of the same genus or members of a very closely related genus. A Euclidian distance of greater than 10 suggests that strain M5A1 and the JD strains are probably not the same species. Strains JD2A5 and JD2J3 are more closely linked, with a Euclidian distance of less than five, suggesting they are of the same species. Strain JD2J12 was linked within a Euclidian distance of 11, indicating a separate species. The bacterial FAME profiles were run in duplicate and the reproducibility was satisfactory; however, none of the JD strains matched any organism presently in the current database (Figure 19). The M5A1 laboratory strain profiles matched that of *Klebsiella* strains in the data base. Also, strain JD9F matched *Pseudomonas* species in the data base as well.

Although there were no perfect matches, strain JD2A5 was very similar biochemically to *Klebsiella* species and was used to generate a plasmid library.

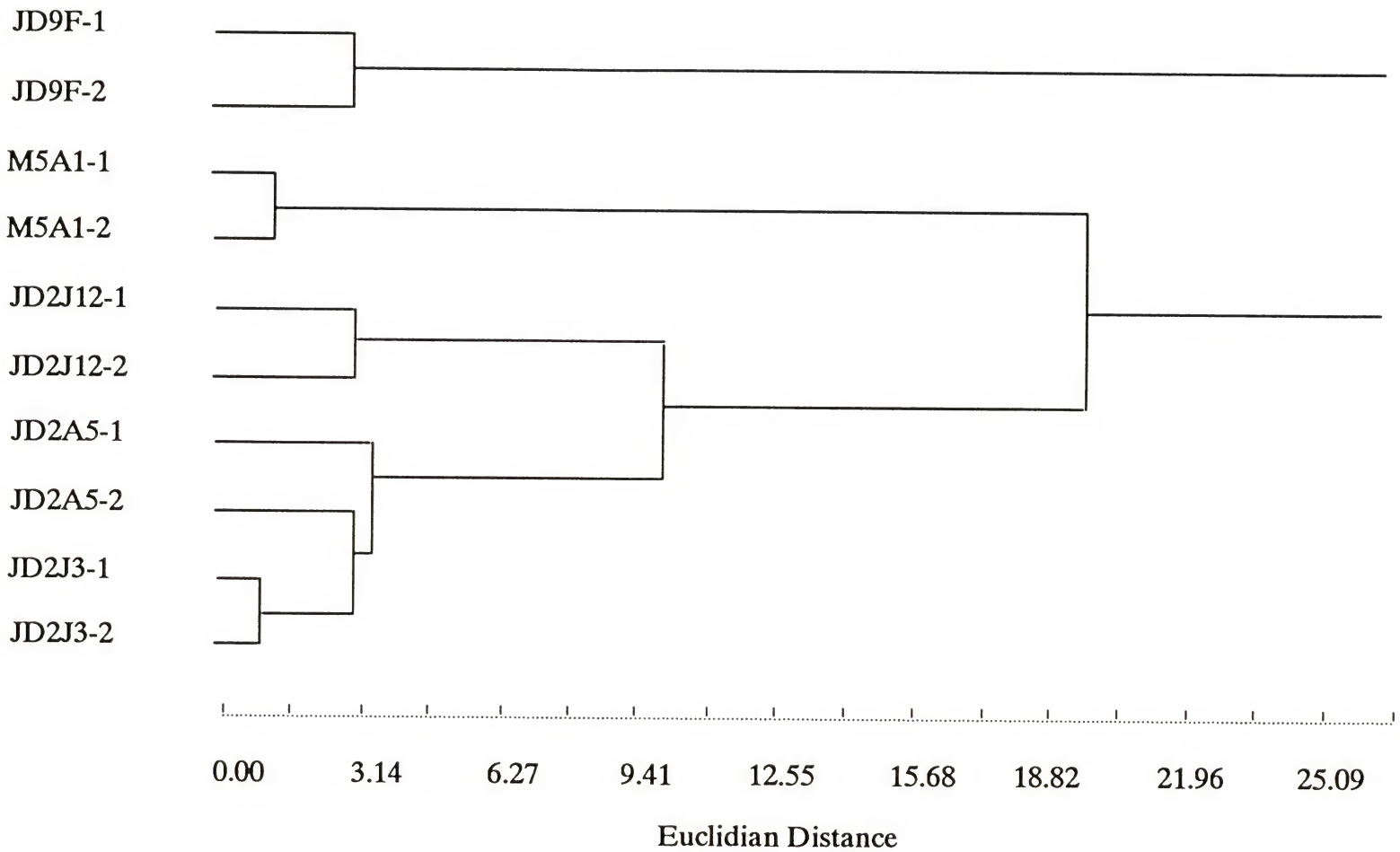


Figure 19. Dendrogram of fatty acid compositions of strains isolated from nature, JD2A5, JD2J3, JD2J12, and JD9F. *Klebsiella oxytoca* strain M5A1 is included for comparison.

Cloning of Genes Encoding CMCase Activity

The plasmid library was screened for clones containing genes encoding CMCase activity. Among the 6000 colonies tested, 24 were identified as CMCase producing colonies. Several with weak activities appeared to be unstable. Restriction enzyme analysis revealed 17 different clones. One of the transformants, pLOI1323, contained a 1.5 kb insert and appeared to be stable in DH5α. Strain DH5α containing the recombinant plasmid, pLOI1323, was negative for MUC, MUG, MUX, MUA, and MUM hydrolyzing activities. However, DH5α (pLOI1323) produced large zones of clearing on starch plates, indicating strong amylase activity (Figure 20). This clone exibited much stronger amylase activity than CMCase activity.

Analysis of DNA sequence

Partial DNA sequencing of pLOI1323 revealed an incomplete open reading frame with a ribosome binding site, GGAAG, 16 bases upstream from an ATG start codon (Figure 21). The deduced amino acid sequence from this region contains 60 % similarity and 45 % identity to an α-glucoamylase precursor from *Saccharomyces cerevisiae* (Figure 22). Homologies to two bacterial hydrolases are also presented in Figure 22.

Discussion

Increased attention to clean air and oxygenates for fuels have intensified the need for processes that can lower the costs associated with ethanol production. These processes can

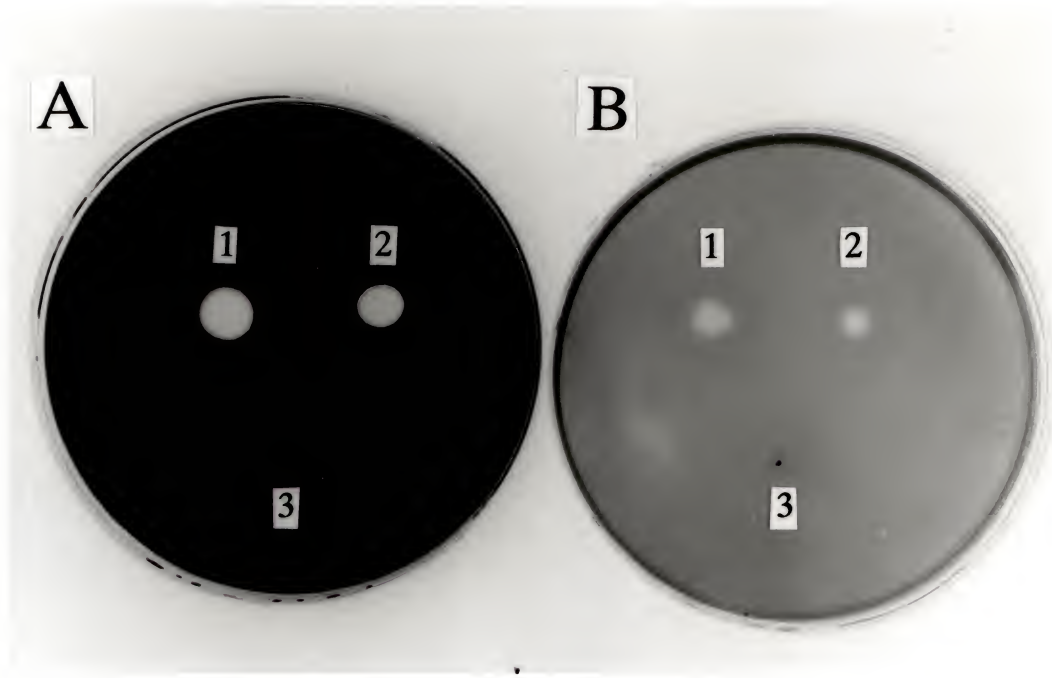


Figure 20. Zones of clearing on a starch indicator plate (A) and carboxymethylcellulose plate (B). Strain JD2A5, 1; *E. coli* strain DH5α containing pLOI1323, 2; DH5α containing plasmid pUC18, 3.

```
1   ACTTAGGCGATGTCTAGCCTTCGAACTCCACTTCGTAAGATGTGAACTTG

51  CGAATATTGATGACGCCAGTATCGAGAATCAGGTATTGCCCTTTAATTCC

101 CTGCAAAATGCCGCTCACTTGTGGCTCTTTATCAAAGTTGTGCGACGTGA

151 TCTTGGTTGGGTAGTGCTCAACCGGATACGCCAGTGAGGTCAAGCTGTCA

201 TGCACGACTTCAACGCTCTCTTCGCCAAACTCGGCTTTTACCTGAGCGAT

251 TTTCTCTTCCAGAAGTGGCAACAATACTTTGGCTTGTTCGGCGAGATCGA

301 GCGGTTGGCCATCACCTTTGAGCAAAGCACGGCAGTTGGTTTTTATCAGC

351 GCATGTGTTTTCGGCAGTTCCACTTCAAGCAAACCGGAAATATGGCGAGT

401 TTTTACCTTCAATATTGGCAAGCTTGCGTCGCACCTTGTCATCAACGGGT
                -35                       -10

451 CGGAAGTTGCGTATGACGTGTGATGCCCCACTTTGAGGCTGGAAGGTGTT
      RB                    M  P  H  F  E  A  G  R  C

501 TAGAAAGGATAAACGAGGTGATCCCCACCCCCGCCAGTTCTGGGACCCAT
    L  E  R  I  N  E  V  I  P  T  P  A  S  S  G  T  H

551 TCAGGTTCCCGACCAGTCCCCGTCGCCTGGTTGCCAGGGTTTTCCGGGCT
     S  G  S  R  P  V  P  V  A  W  L  P  G  F  S  G  L

601 TTCAAACATGGGCCGATCTTCGCCAGGCTCGGGGCCATTTTTGGCTTCAA
      S  N  M  G  R  S  S  P  G  S  G  P  F  L  A  S

651 CACAGAGTTCATCAGTCCCATTAGGGTTAGCTGCT
    T  Q  S  S  S  V  P  L  G  L  A  A
```

Figure 21. Partial nucleotide sequence of the 1.5-kbp fragment from JD2A5 contained in pLOI1323. The deduced amino acid sequence is listed below the first nucleotide of the corresponding codon. A putative promotor region for this gene is underlined, with the -10 and -35 regions labeled.

A. Glucoamylase

```
1323          -MPHFEAGRCLERINEVIPTPASSGTHSGSRPVP----------VAWLPGFSGLSNMGRSSPGSGPFLASTQSSSVPLGLAA
SC-S1         APVPTPSSSTTESSSAPVPTPSSSTTESSSAPVPTPSSSTTESSVAPVPTPSSSSNITSSAPSSTPFSSSTESSSVPVPTPS
                   .:.. *. ...: **.**.*.*:* **          ** :*. *: **:..*.*:*.** .**:*****:. :.
```

B. Pullulanase

```
1323          MPHFEAGRC------LERINEVIPTPASSGTHSGSRPVPVAWLPG--------------------FSGLSNMGRS--SPGS
KA-PulA       LYLWNNETCDALSAPVADWNDVSTTPTGSDKYGPYWVIPLTKESGCINVIVRDGTNKLIDSDLRVSFSDFTDRTVSVIAGNS
              :  ::.: *      :.  *:* .**.:*:..:. :.:*:.. .*                    **:.:.:.. *  ...*

              GPFLASTQSSSVPLGLA
              AVYDSRADAFRAAFGVA
              :.: ...:. ..::*:*
```

C. Amylopullulanase

```
1323          FEAGRCLERINEVIPTPASSGTHSGSRPVPVAWLPGFSGLSNMGRSSPGSGPFLASTQSSS----------VPLGLA
B-AapT        FKNVSSIRNNNQVLKTGDLETLYAQNDVYAIGR-RIINGKDAFGNSYPDSAAIVAINRSNSDQQITIDTTKFLRDGVA
              *....:: . *:*:.*.. .. .... . :::: . :.* ..:* * *:*::::* ..*.*          :. *:*
```

Figure 22. Comparison of deduced amino acid sequences of pLOI1323 (incomplete open reading frame) with other hydrolytic enzymes. A. Glucoamylase precursor S1 protein from *Saccharomyces cerevisiae* (glucan 1,4-α-glucosidase) (60% similarity, 45% identity). B. *Klebsiella aerogenes* pullulanase (PulA) gene product (50% similarity, 20% identity). C. *Thermoanaerobacterium saccharolyticum* amylopullulanase gene product (AapT)(47% similarity, 21% identity). Asterisks and dots indicate identity and similarity, respectively.

in turn improve the competitiveness of ethanol as a fuel or fuel additive. *Klebsiella oxytoca* strain P2 was specifically developed for use in a simultaneous saccharification and fermentation process. If cellulase genes could be cloned into this organism and expressed to adequate levels, the need for supplementary fungal enzymes may be reduced. At present, it is the addition of fungal cellulases that makes up a large portion of the cost of a bioconversion process.

Carboxymethylcellulose has been used by many researchers as a simple method to identify endoglucanase activity. It was shown that strain JD2A5 and *E. coli* strain DH5α (pLOI1323), both produced clear zones of hydrolysis on CMC agar. However, hydrolyzed products from carboxymethylcellulose could not be detected when measuring reducing sugars liberated into the reaction mixture. When starch was used instead of CMC, and all other conditions held constant, reducing sugars were detected (data not shown). Recently, Lee and colleagues (1994) have observed this same phenomenon where a cloned gene from *Bacillus* strain XAL601 exibited zones of clearing on indicator plates containg CMC, xylan, starch and pullulan. However, hydrolyzed products from xylan and CMC could not be detected. They concluded that the zones of clearing may not have been formed by hydrolysis but possibly only by the binding of enzyme and substrate.

Consequently, it would appear that we have cloned a gene whose main activity is amylolytic rather than cellulolytic.

Amylases are used in starch processing industries where it is desirable to hydrolyze α-1,4-glucosidic linkages to produce a mixture of glucose, maltooligosaccharides, and α-limit dextrans. Nevertheless, in the process of converting cellulose to a form that is useful for bacterial cells, it is a ß-1,4-glucosidic linkage that is the target for hydrolysis.

Alternatively, a naturally cellulolytic organism could perhaps be metabolically engineered to produce ethanol as its main product while maintaining its ability to produce active cellulases.

Klebsiella-like strain JD2A5 appeared to be a likely candidate for transformation using the alcohol pathway from *Zymomonas mobilis* utilizing protocols previously described (Ohta et al., 1991; Wood and Ingram, 1992). However, attempts to introduce foreign DNA into this strain were unsuccessful. Several transformation protocols, conjugation protocols, and electroporation methods were tried to no avail.

In conclusion, strain JD2A5 isolated from a cellulose plant is very similar to *Klebsiella* species and contains at least one very active amylase. What is not clear, however, is whether or not this strain exibits any true cellulolytic activity. It may be that screening on CMC agar selects for organisms that contain CMC binding enzymes that may or may not actually display hydrolytic activity. This organism, or genes cloned from this organism, may be useful in starch conversion processes. However, it is doubtful that the recombinant

plasmid, pLOI1323, can be used in a cellulose-to-ethanol process, even if the CMCase activity observed on plates is true hydrolytic activity. When *K. oxytoca* strain P2 was transformed with pLOI1323, plasmid stability was diminished compared to that of DH5α containing the same plasmid.

For these reasons, further analyses of the JD2A5 strain or the recombinant plasmid pLOI1323, were discontinued. Since many genes involved in cellulose hydrolysis have been cloned and sequenced, it may prove beneficial to screen known enzymes for cellulolytic activity in *K. oxytoca* strain P2, in addition to isolating new cellulase genes.

CHAPTER V
SCREENING OF THREE BACTERIAL CELLULASES FOR USE IN SIMULTANEOUS SACCHARIFICATION AND FERMENTATION EXPERIMENTS USING *KLEBSIELLA OXYTOCA* STRAIN P2

Introduction

Plant biomass is an abundant renewable resource that can serve as a substrate for the production of alternative fuels, such as ethanol (Vallander and Eriksson, 1990). Potentially, both the hemicellulose and cellulose can be solubilized and fermented to ethanol. Dilute acids have been used to effectively hydrolyze the hemicellulose component, while improving the digestability of the cellulosic residue (Grohmann and Himmel, 1991). Sugars generated from hemicellulose in this fashion have been efficiently fermented to ethanol by genetically engineered *E. coli* containing genes encoding the alcohol pathway from *Zymomonas mobilis* (Barbosa et al., 1992; Han-Hagerdal et al., 1993; Beall et al., 1992; Lawford and Rousseau, 1991; Lawford and Rousseau, 1993). The cellulose component has also been converted into ethanol by a separate, simultaneous saccharification and fermentation process (SSF) using commercially available fungal cellulase with *K. oxytoca* strain P2 as the biocatalyst (Chapter II; Doran and Ingram, 1993; Chapter III; Doran and Ingram, 1994). Strain P2 contains transport systems and hydrolases which

allow this organism to metabolize cellobiose and cellotriose, eliminating the need for fungal ß-glucosidase (Wood and Ingram, 1992). In an optimal process, both the hemicellulose and the cellulose components could be utilized by recombinant strains for the production of ethanol. *K. oxytoca* strain P2 or *E. coli* strain KO11 could be used to ferment the pentose and hexose sugars generated from hemicellulose hydrolysis of sugar cane bagasse, corn cobs, corn stover, or other agricultural residue. At the same time, endoglucanase could be produced intracellularly within the recombinant bacteria during pentose fermentation. This cell mass could then be harvested and added to the agricultural residue as a supplemental source of cellulase enzyme in a separate SSF of the cellulose-rich residue. In this fashion, the total cost of a bioconversion process may be reduced by decreasing the amount of fungal enzyme required for the SSF process.

Many bacteria and fungi participate in the recycling of carbon photosynthetically-fixed in plant matter. The cellulolytic fungus, *Trichoderma longibranchiatum* (formerly *T. reesei*), has long been recognized as one of the most efficient cellulose-degrading microorganisms (Reese, 1976). Hyperproducing mutants have been selected that are insensitive to catabolite repression, constitutive for cellulase synthesis, and exhibit increased ß-glucosidase production (Durand et al., 1988). Currently, the rates of protein synthesis obtained with these mutants is thought to approach

the theoretical limit for a biological system. Although further modifications of the productivity of this organism may help decrease the cost of the cellulases, major cost reductions must come from other areas (Beguin et al., 1988a). The economic feasibility of enzymatic cellulose degradation is also hampered by the low specific activity of the cellulase enzymes (Beguin et al., 1988a).

Commercially available preparations from *T. longibranchiatum* have been used successfully in simultaneous saccharification (SSF) experiments (Chapter II; Gauss et al., 1976; Blotkamp et al., 1978; Takagi et al., 1977; Freer, 1991, Spindler et al., 1992; Doran and Ingram, 1993; Doran and Ingram, 1994). However, the cost of cellulase enzymes remains a major contributor to the final cost of ethanol. One possible solution to this problem would be to discover enzyme systems with specific activities which are higher than *T. longibranchiatum*. However, such systems must secrete very high levels of cellulase enzymes. Alternatively, the efficacy of commercial *T. longibranchiatum* preparations may be improved by supplementation with enzymes from other systems with high specific activities. It is possible that the addition of bacterial enzymes with high specific activities could reduce the amount of fungal enzyme needed in an SSF process.

Clostridium thermocellum is a Gram-positive, spore forming, obligately anaerobic bacterium with an optimum growth temperature between 55-60°C. Specific activity of the

cellulase system produced by this organism is one of the highest known for crystalline cellulose (Johnson et al., 1982). The cellulase system of *C. thermocellum* forms an extracellular multienzyme complex with a M_r of 2.1 million. This complex, termed a cellulosome, is comprised of 14-18 different polypeptides and is present in the culture medium and on the cell surface (Lamed et al., 1983). Several groups have been involved in the cloning of *C. thermocellum* cellulase genes (Cornet et al., 1983; Millet et al., 1985; Swartz et al., 1985; Romaniec et al., 1987; Kadam et al., 1988; Wu et al., 1993). *C. thermocellum* endoglucanase D was purified from an *E. coli* hyperproducing clone containing a fusion between the coding sequence of *celD* and the 5' terminal region of *lacZ* contained in plasmid vector pUC8 (Joliff et al., 1986). Although it is difficult to compare CMCase activities reported by different laboratories, endoglucanase D (EGD) appears to be one of the most active cellulases described. For example, an activity of 428 μmoles of glucose equivalents per mg protein was reported for EGD (Joliff et al., 1986) versus 65 μmoles of glucose equivalents per mg protein for endoglucanase I of *T. reesei* (Deshpande et al., 1984). The activity of *C. thermocellum* endoglucanase A was reported to be 117 μmoles of glucose equivalents per mg protein (Fierobe et al., 1991), and 59 μmoles of glucose equivalents per mg protein for a cellulase isolated from an alkalophilic *Bacillus* sp. (Fukumori et al., 1986).

Although many genes involved in cellulose depolymerization have been isolated, it is likely that there are many environmentally important cellulolytic organisms whose nutritional requirements have not been met by traditional laboratory techniques (Stewart et al., 1988). Recently, Healy and coworkers (1995) cloned several cellulase genes using DNA isolated directly from the sludge in thermophilic anaerobic biomass digesters. The nucleotide sequence of the DNA insert from one of these plasmids, pFGH1A, was determined and found to contain a novel cellulase, 332 amino acids long. This cellulase shared limited homology with members of the A3 subfamily of cellulases which includes *C. thermocellum* endoglucanase C (EGC) (40% identity).

A subclone containing the cellulase gene, pFGH1A, along with a second clone from the anaerobic digester, designated pFGH12A, and *C. thermocellum* endoglucanase D were evaluated in an SSF process using a fungal cellulase preparation and *Klebsiella oxytoca* strain P2 as the biocatalyst.

Materials and Methods

Bacterial strain, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 6. Ethanologenic strains *K. oxytoca* strain P2 and *E. coli* strain KO11 were grown at 30°C in Luria broth (Sambrook et al., 1989) supplemented with 50 g glucose per liter, or Luria agar with 20 g glucose per liter. *E. coli* strain DH5α was grown at 37°C. Ampicillin (50μg ml^{-1}) was

Table 6. Strains and plasmids used in this study.

Strain or plasmid	Relevant Genetic Trait(s)	Reference
Strains		
K. oxytoca P2	*adh pdc* from *Z. mobilis*	Wood and Ingram, 1992
E. coli KO11	*adh pdc* from *Z. mobilis*	Ingram et al., 1991
E. coli DH5α	Δ*lacZ*M15 *recA*	BRL
Plasmids		
pFGH1A	pUC18 containing *cel* gene from anaerobic digester	Healy et al., 1995
pFGH12A	pUC18 containing *cel* gene from anaerobic digester	Healy et al., 1995
pCT603	pUC8 containing *celD* gene from *Clostridium thermocellum*	Joliff et al., 1986

added to media after sterilization as appropriate for selection of recombinant organisms.Determination of Carboxymethylcellulase (CMCase) Activity

Colonies were initially screened for CMCase activity by the Congo red method using Luria agar supplemented with 0.2% carboxymethylcellulose (Beguin, 1983). CMCase activity was measured using the method of Beguin et al. (1988b). One unit of activity corresponds to the amount of enzyme required to produce 1 nmol glucose equivalent per minute. Specific activity is expressed in units per milligram protein. Protein concentrations were estimated using the enhanced alkaline copper (Lowry) protein assay with bovine serum albumin as a standard (Stoscheck, 1990). All assays were conducted in triplicate and reported as an average value.

Zymograms

Cell extracts of KO11 carrying various plasmids were prepared as previously described (An et al., 1991) with one modification. Extracts were not centrifuged to remove the membranes. Although protein samples were not denatured by boiling, SDS was included in the buffers as suggested by Cavedon et al., (1990). Samples were incubated at room temperature for 15 minutes in running buffer (Laemmli, 1970) containing 2% SDS (wt/vol) prior to loading. Electrophoresis was performed in the presence of SDS (0.1% wt/vol) by the method of Laemmli (1970) in a Mini Protean II System (Bio-Rad). Gels were stained with Coomassie brilliant blue R-250.

The zymogram technique of Coughlan (1988) was used to locate CMCase activity in these gels.

DNA Sequencing and Analysis

Double-stranded plasmid DNA was purified for sequencing by utilizing Magic Mini Prep columns (Promega Corp., Madison, WI). Sequencing of the pFGH12A DNA insert was carried out using a LI-COR (Lincoln, NE) model 4000 DNA sequencer by the Dept. of Microbiology and Cell Science DNA Sequencing Facility (Sanger et al., 1977). Resulting sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereux et al., 1991) and the National Center for Biotechnology Information database searches using BLAST network server (Altschul et al., 1990). Amino acid sequences were aligned by the CLUSTAL V program (Higgins et al., 1992).

Fermentation Experiments

Preparation of inocula and media have been previously described (Chapter II; Doran and Ingram, 1993). Sigmacell 50 crystalline cellulose was used as the substrate at a final concentration of 100 g liter^{-1}. In these studies, bacterial cellulase was tested as a supplemental activity in SSF experiments which contained the commercial fungal cellulase, Spezyme CP. Spezyme CP cellulase was provided by Genencor International (South San Francisco, CA) and was reported to contain approximately 100 filter paper units of activity ml^{-1}. It was sterilized by filtration prior to use and added at a

final concentration of 5 FPU g^{-1} cellulose at the time of inoculation.

Preparation of Recombinant Cellulase

The predominant sugar in hemicellulose hydrolysates of agricultural residue is xylose. Therefore, xylose was used as the substrate for growth of recombinant *E. coli* strain KO11. Strain KO11 containing plasmids with cellulase genes was grown for 24 h at 30°C in a shaking water bath in Luria broth containing 90 g xylose $liter^{-1}$ and ampicillin (50μg ml^{-1}). Strain KO11 containing pCT603 required 36 h for adequate growth versus 24 h for the other recombinants.

Results

DNA Sequencing and Analysis

E. coli strain KO11 containing plasmid pFGH12A exibited CMCase activity in liquid and plate CMCase assays. In order to further characterize this activity, the 1.5-kbp fragment was sequenced. One complete open reading frame encoding a protein of 250 amino acids was found (Figure 24). This 750-bp open reading frame encoded a protein with an apparent anhydrous M_r of 27,390. The deduced amino acid sequence exhibited limited homology to the amino terminal regions of *Clostridium cellulolyticum* endo-ß-1,4-glucanase (CELCCD) (54% similarity, 37% identity), and *Ruminococcus albus* ß-1,4-D-glucanase (CELA) (53% similarity, 27% identity) (Figure 25). Limited homology was also observed to a more central region of

```
   1  TCGAGCTCGG TACCGGCTTT GACGACAATT TATCAACCAC GTTGGGAACT
  51  TGGTCAGATT TTTTGTCAGT GACTCATAGA AAGGGTTACG GGGCATGGAC
 101  CGAAAATCCC TAGACATGTT GTGCTTCCAA CGACACTAAT CGACAGACAA

 151  TCTGTCAGAG ACATCGAAAA TGATATTTGA GATGATTGTC AGGAGATGAA
                                                    SD
 201  AATAGCCATG AGAACGTATT TGTGGCTTTT GGTATTCTTA CTGGTTGCCG
           M   R  T  Y  L   W  L  L   V  F  L   L  V  A  V
 251  TAGTGGGAGG GATTATAGTG TCTGATAAAC TCGACAGTAG ACATGATGTC
        V  G  G   I  I  V   S  D  K  L   D  S  R   H  D  V
 301  AATAATGGTA ATGGTGATGA ATTGAGAACG TCAAGCATAT TTGATTACAA
      N  N  G  N   G  D  E   L  R  T   S  S  I  F   D  Y  N
 351  CAAGAAAATT GGTCACGGAG TTAACATAGG TAATGCCCTT GAGGCACCCG
        K  K  I   G  H  G  V   N  I  G   N  A  L   E  A  P  V
 401  TTGAAGGTTC TTGGGGGGTT TACATATCGG ATGATTATCC CCCTCTCATC
        E  G  S   W  G  V   Y  I  S  D   D  Y  P   P  L  I
 451  AAGGAACGTG GCTTTAATTC TGTACGTATT CCCATTCGCT GGTCAGCTCA
      K  E  R  G   F  N  S   V  R  I   P  I  R  W   S  A  H
 501  CATCGAAGAT AATCATCCGT ACAAGATAGA TGAGGAGTTC TTAAATAGAG
        I  E  D   N  H  P  Y   K  I  D   E  E  F   L  N  R  V
 551  TAAAACACGT TGTTGATAAA GCGTTGGAAA ACAACTTAAT TGTCGTCATA
        K  H  V   V  D  K   A  L  E  N   N  L  I   V  V  I
 601  AACACACACC ACTTTGAAGA AATGTATCAA TCACCCGAAG AGTACGAAGA
      N  T  H  H   F  E  E   M  Y  Q   S  P  E  E   Y  E  D
 651  CAGACTGGTG GAGATCTGGA GGCAAATATC CGACACGTTT AAAAATTATC
        R  L  V   E  I  W  R   Q  I  S   D  T  F   K  N  Y  P
 701  CCAACACTCT CTACTTCGAG ATATTCAACG AGCCTGCACA GAATTCGACT
        N  T  L   Y  F  E   I  F  N  E   P  A  Q   N  S  T
 751  CCAGAGCTGT GGAATGATAT CTATCCTCAA GCGTCGAATA TAGTACGCGA
      P  E  L  W   N  D  I   Y  P  Q   A  S  N  I   V  R  D
 801  CACGAATCCA AATAGGGTGG TAATCATCGA TGTTCCACAT CCGTCGACCA
        T  N  P   N  R  V  V   I  I  D   V  P  H   R  S  T  I
 851  TTCAGCATCG ACGACTGAAG CTCGTAAACC GAACACACAT CGTCCTCTTC
        Q  H  R   R  L  K   L  V  N  R   T  H  I   V  L  F
 901  ATTACTACGA CCATCGCATT ACACACAGCG CAGATGGTCC TCACACACCG
      I  T  T  T   I  A  L   H  T  A   Q  M  V  L   T  H  R
 951  AGGGTAGTTA CAATCACGCC ATGCCATTGT AGTATGGCCA GGAACATTGA
        G  *
1001  CCATCTTCCG GTAATCGGGC TTATCAGGGC AGATATGGAA TCTCCCGCTT
1051  ATGACAGAGC AGTTAGAAGA CAGCCGAAAA TCGGTTTTTC ACTGCATATT
1101  GGGATTTGTC TGGTTTGGTC TCTACGATAC CTCATCGAAC AAATGGCTTG
1151  ACCTTTGACT ACTTCAGCAC TTGGAAGATA GTGCGATTCA ATAGAAGTTA
1201  ATAGAAGAAC AATAAATGAA TGGATTTCAA AACAGAGAGG GAGGGAGTTT
1251  GAACGTGATA GTTTACGGCA CGGAGTTGAA AAACATACCT TGGGAAGAGA
1301  GGCCAGCAAG TTGTAGTGAT GTTTTGTGGA GGTATTCAAA TAACCCAATA
1351  ATAAAGCGAA ATCAAGCAAA GGATGCAAAT AGTATATTCA ACAGTGCGGT
1401  TGTACCATTC AACGGTGAGT TTGCGGGTGT ATTCAGGGTT GATGATAGAC
1451  AAAGAAGGAT GAATGTGAGA AGAGGATTCA GTAAAGATGG TTTAAATTGG
```

Figure 24. Nucleotide sequence of the 1.5-kbp DNA insert in pFGH12A. The deduced amino acid sequence is listed below the first nucleotide of the corresponding codon. An asterisk marks the stop codon terminating this gene. A "Shine-Dalgarno" (SD) region for this gene is underlined.

Figure 25. Comparison of deduced amino acid sequence of the ORF in plasmid pFGH12A (FGH12A) with other endoglucanases. A. *Clostridium cellulolyticum celCCD* gene product (CTCelCCD) (54% Similarity, 37% Identity). B. *Clostridium thermocellum celH* gene product (CTCelH)(51% Similarity, 32% Identity). C. *Ruminococcus albus celA* gene product (RACelA) (53% Similarity, 27% Identity). Vertical lines and dots represent identity and similarity, respectively. Numbers to the right represent the position of the rightmost amino acid in the respective protein.

```
A.FGH12A    KKIGHGVNIGNALEAPVEGSWGVYISDDYPPLIKERGFNSVRIPIRWSAHIEDNHPYKIDEE 110
CCCelCCD    KKMGIGMNLGNTFDAPTEGSWSKAAQEYYFDDFKQAGFKHVRIPIRWDQHTLANSPYTVDSN  65

            FLNRVKHVVDKALENNLIVVINTHHFEEMYQSPEEYEDRLVEIWRQISDTFKNYPNTLYFEI 172
            FLNRIETVIDWSLSRGFVTVINSHHDTWLMDNYSQNIGRFEKIWEQIAQRFKGKSENLVFEI 126

            FNEPAQNSTPELWNDIYPQASNIVRDTNPNRVVIIDVPHRSTIQ.HRRLKLVNRTHIVLFI. 232
            LNEPHGNITDSQINDMNKRILNIIRKTNPTRNVIIGAGYWNSYNSLSQLEIPNDPNLIATFH 188

            ..............TTTIALHTAQMVLTH                                 247
            YYDPYSFTHQWQGTWGTKNDMDAIAMVFNH                                218

B.FGH12A    LWLLVFLLVAVVGGIIVSDKLDS.....................................  39
CTCelH      VFSRAYQALASINKPIIIAEFASAEIGGNKARWITEAYNSIRTSYNKVIAAVWFHENKETDW 342

            .............RHDVNNGNGDELRTSS.............IFDYNKKIGHGVNIGNALEA  75
            RINSSPEALAAYREAIGAGSSNPTPTPTWTSTPPSSSPKAVDPFEMVRKMGMGTNLGNTLEA 404

            PVEGSWGVYISDDYPPLIKERGFNSVRIPIRWSAHIEDNHPYKIDEEFLNRVKHVVDKALEN 137
            PYEGSWSKSAMEYYFDDFKAAGYKNVRIPVRWDNHTMRTYPYTIDKAFLDRVEQVVDWSLSR 466

            NLIVVINTHHFEEMYQSPEEYEDRLVEIWRQISDTFKNYPNTLYFEIFNEPAQNSTPELWND 199
            GFVTIINSHHDDWIKEDYNGNIERFEKIWEQIAERFKNKSENLLFEIMNEPFGNITDEQIDD 500

            IYPQASNIVRDTNPNRVVIIDVPHRSTIQHRRLKLVNRTHIVLFITTTIALHTAQMVLTHRG 261
            MNSRILKIIRKTNPTRIVIIGGGYWNSYN..TLVNIKIPDDPYLIGTFHYYDPYEFTHKWRG 562

C.FGH12A    MRTYLWLLVFLLVAVVGGIIVSDKLDSRHDVNN.........................GN  35
RACelA      LVTLITVLAMASSGLVSCGPGNGKDSSQAEKKDTSSAAESTADSDAQPAGDTTLLSNEELAR 139

            GDELRTSSIFDYNKKIGHGVNIGNALEAPVEGSWGVYISDDYPPLIKE.......RGFNSVR  90
            SGEVRDISAMELVAEMKTGWNLGNSLDATGAAGNASEVNWGNPKTTKEMIDAVYNKGFDVIR 201

            IPIRWSAHIEDNHPYKIDEEFLNRVKHVVDKALENNLIVVINTHHFEEMYQSPEEY....ED 148
            IPVTWGGHVGDGPDYKIDENWLARVQEVVNYAYDDGAYVIINSHHEEELRIPDNEHIDAVDE 263

            RLVEIWRQISDTFKNYPNTLYFEIFNEPAQNSTPELWNDIYPQASNIVRDTNPNRVVIIDVP 210
            KTAAIWKQVAERFKDYGDHLIFEGLNEPRVKGSPEEWNGGTEEGRRCV..ERLNQTFLDTVR 323

            HRSTIQHRRLKLV...NRTHIVLFITTTIALHTAQMVLTHRG                     249
            ATGGNNEKRLLLMTTYASSCGLKLIQDTAIPEDDHIGFSIHA                     365
```

Clostridium thermocellum CELH protein (51% similarity, 32% identity) (Figure 25).

Recombinant Cellulase Activity

Plasmids were initially transformed into *K. oxytoca* strain P2 and *E. coli* strain KO11. However, these plasmids were relatively unstable in strain P2. Their use in this organism was not pursued further. A comparison of carboxymethyl-cellulase activities of *E. coli* strain KO11 carrying various plasmids is shown in Table 7. French press extracts of overnight cultures were used as crude enzyme preparations by analyzing CMCase activity. Enhanced CMCase activity was observed when the culture was supplemented with ampicillin (50 μg ml^{-1}) halfway through the growth period. The values in Table 7 represent bacterial preparations assayed in triplicate under optimal conditions (pH 6.0 and 55°C). The commercial *T. longibranchiatum* cellulases, Spezyme CE and CP, were approximately 10 times higher than the best bacterial preparation KO11(pCT603), 154 times higher than KO11 (pFGH12A), and 684 times higher than KO11 (pFGH1A). Spezyme CP cellulase is a proprietary improvement over Spezyme CE cellulase which had been used in previous studies. The Spezyme CP preparation contained more CMCase activity and was used in these SSF experiments.

Examination of proteins in crude extracts used for CMCase assays failed to show any significantly overexpressed protein bands (Figure 26). To locate CMCase activities, the

Table 7. Comparison of carboxymethylcellulase activities of *E. coli* strain KO11 carrying different plasmids.

plasmid	CMCase activity[a,b]
pFGH1A	1
pFGH12A	58
pCT603	890
pUC18	0
Spezyme CP cellulase	10,201
Spezyme CE cellulase	7,670

[a] CMCase activity is presented as nmol min^{-1} per mg protein.
[b] Enzyme assays were conducted under optimal conditions: recombinant proteins , pH6.0 and 55°C; Spezyme CE and CP cellulases, pH 4.8 and 48°C.

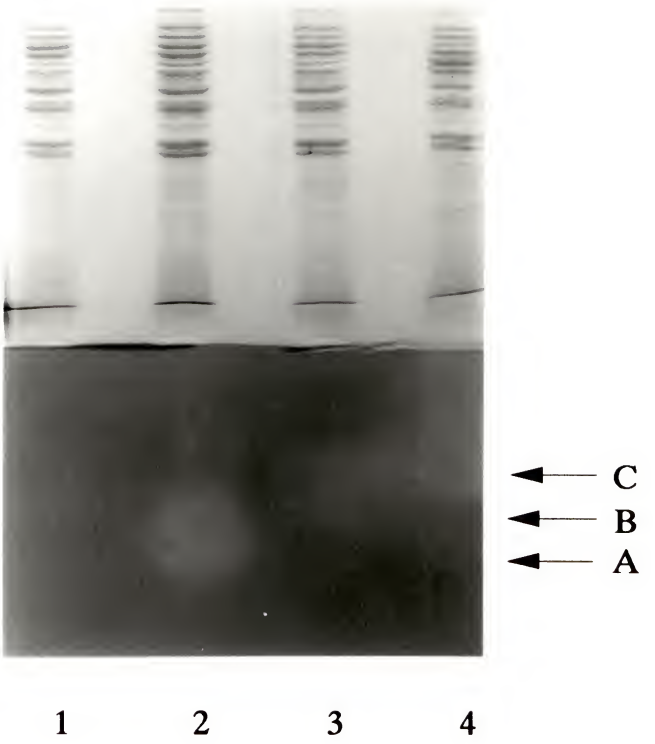


Figure 26. Upper gel: Protein gel stained with Coomassie Brilliant Blue R-250. Each lane contains approximately 20 μg of protein per lane. Lower gel: Zymogram stained for CMCase activity. Lanes for both gels (protein loaded for zymogram) lane 1, pUC18 (120 μg); lane 2, pFGH12A (40 μg); lane 3, pFGH1A, (120 μg); and lane 4, pCT603 (10 μg). The gel and overlay were incubated for 7 h at 60°C before staining with Congo red.

polyacrylamide gels were incubated for varying times at 60°C with a CMC agar overlay (Coughlan, 1990) (Figure 26). Distinct zones of hydrolysis were observed in lanes 2 and 4, indicated by letters A and C, respectively. The zone of hydrolysis generated from pFGH1A in lane 3 is indicated by the letter B. The high activity of pCT603 (lane 4) makes observation of the pFGH1A (lane 3) zone somewhat difficult.

Fermentation Experiments

Recombinant cellulase produced in *E. coli* strain KO11 was tested for its ability to supplement fungal cellulase in an SSF process. Strain KO11 containing plasmids with and without cellulase genes was harvested by centrifugation and added to SSF experiements at a final concentration of 1.65 g dry weight $liter^{-1}$. Fermentation vessels (Fleakers) were equilibrated at 60°C for 1 h before the cell pellets were added. Cell pellets were resuspended in the Sigmacell 50-containing fermentation broth and incubated at 60°C for 1 h to facilitate cell lysis and release of bacterial enzymes. Fleakers were then cooled to 35°C, adjusted to pH 5.2, and inoculated with *K. oxytoca* strain P2 at an initial cell density of 330 mg dry weight $liter^{-1}$. Fermentations were conducted as previously described (Chapter II; Doran and Ingram, 1993). Results presented are an average from three or more experiments (Figure 27; Table 8). Bacterial enzyme improved SSF in all cases as compared to the control with KO11 containing unmodified pUC18 or KO11 alone. KO11 (pCT603) containing *C. thermocellum celD* gene was

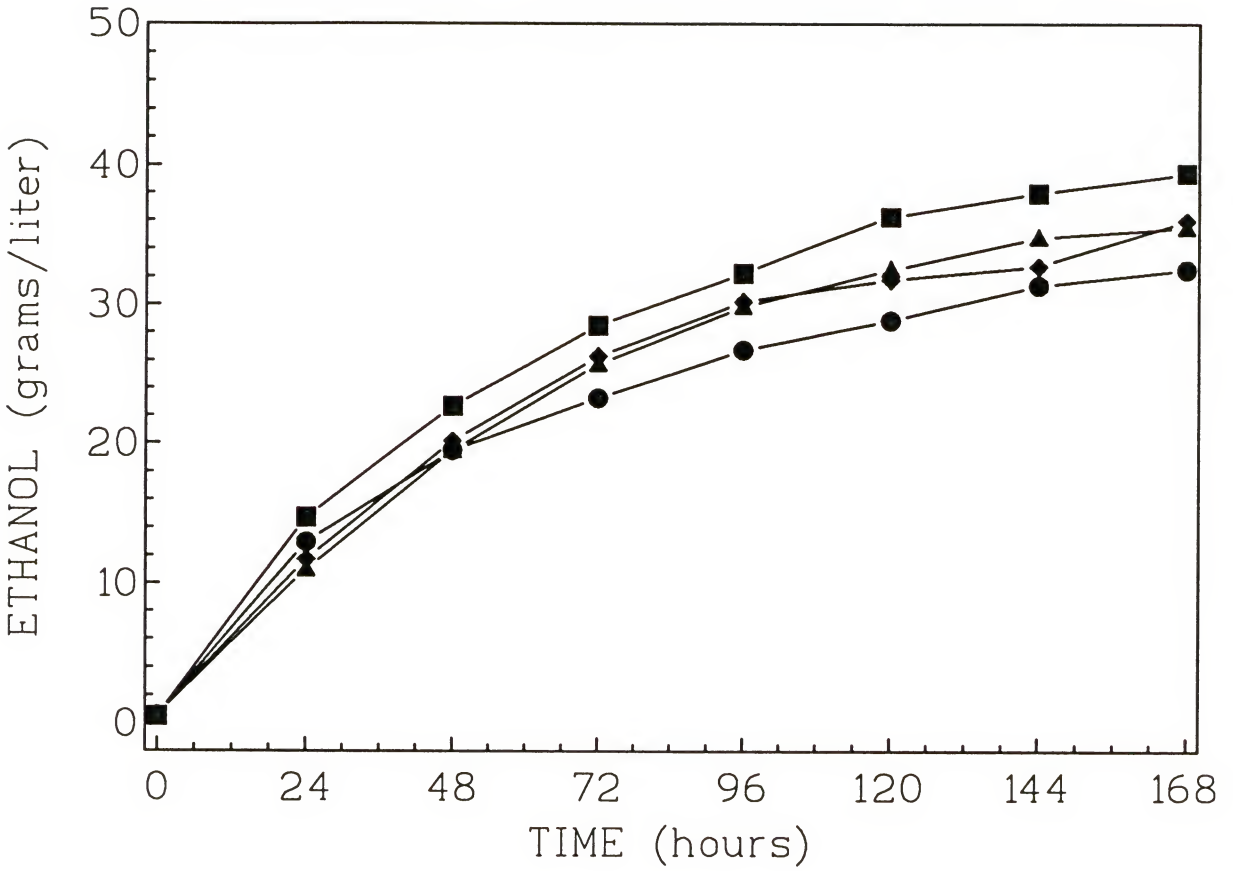


Figure 27. Ethanol production by *K.oxytoca* P2 strain (as described in the text) using *E.coli* strain KO11 containing various plasmids to supply additional cellulase. ■, pCT603; ▲, pFGH12A; ♦, pFGH1A; ●, pUC18.

Table 8. Comparison of ethanol production from Sigmacell 50 (100 g liter^{-1}) and Genencor Spezyme CP cellulase (5 FPU g^{-1} cellulose) supplemented by strain KO11 containing plasmids encoding bacterial cellulase.

Plasmid	Replicates	Maximum Ethanol ± SD[a] (g liter^{-1})	Theoretical[b] Yield (%)	Yield (g ethanol g^{-1} cellulose)
pUC18	6	32.5 (1.7)	57	0.33
pCT603	3	39.4 (0.8)	71	0.40
pFGH1A	6	36.0 (0.6)	64	0.36
pFGH12A	3	35.5 (0.6)	63	0.36

[a] SD, standard deviation, values presented in parentheses.
[b] Maximum theoretical yield for cellulose is 0.568 g ethanol g^{-1} cellulose. Yields were calculated with correction for base addition.

the best of the three tested. SSF experiments with this plasmid produced ethanol more rapidly and reached higher ethanol concentrations than fungal cellulases alone. KO11 (pCT603) reached maximum ethanol values of 39.4 g ethanol liter^{-1}, exhibiting an increase of over 20 % in ethanol yield (Table 8). KO11 containing plasmids pFGH1A and pFGH12A were nearly identical (36.0 g ethanol liter^{-1} and 35.5 g ethanol liter^{-1}, respectively). Both provided an increase of approximately 10% over ethanol production values for controls containing no plasmid or plasmid pUC18.

Discussion

The SSF process using recombinant *K. oxytoca* appears to offer many advantages in performance, based on comparisons of reported enzymatic conversions of cellulose to ethanol. Spindler et al. (1992) have reported the best yeast-based conversions of cellulose to ethanol using Sigmacell 50 cellulose as a substrate and Genencor cellulase (product 150L, no longer available). In these studies, *Brettannomyces custersii* produced 32 g ethanol liter^{-1} (76 % of theoretical yield) in 72 hours from 75 g cellulose liter^{-1} and 26 FPU cellulase g^{-1} cellulose. With higher levels of cellulose (100 g liter^{-1}) and slightly lower amounts of cellulase (19 FPU g^{-1} cellulose), yields were reduced to 71 % of theoretical (40 g ethanol liter^{-1}) and required 240 h for completion. Analogous fermentations using *K. oxytoca* P2 strain, Sigmacell 50 cellulose (100 g liter^{-1}), and Genecor Spezyme CE cellulase at

about half the enzyme level used in the yeast-based studies (10 FPU g^{-1} cellulose) produced 42 g ethanol $liter^{-1}$ (74 % of theoretical yield) after 168 h (Doran and Ingram, 1993). An enzyme loading of 10 FPU g^{-1} cellulose appeared to approach saturation, since ethanol concentrations obtained from higher loadings resulted in only modest further increases in ethanol. Fermentations using enzyme loadings of 5 FPU of Spezyme cellulase g^{-1} cellulose reached 57 % of theoretical yield (32.5 g $liter^{-1}$).

In order to evaluate the contribution of the recombinant bacterial cellulases to the production of ethanol, further experiments were conducted with an enzyme loading of 5 FPU of g^{-1} cellulose. Although different preincubation temperatures and pH conditions were examined (data not shown), the ethanol values obtained were essentially the same. The maximum ethanol concentrations were reached at 168 h for all fermentations. These values are presented in Table 8 along with the number of experiments and the standard deviations. *E. coli* strain KO11 without plasmid and with plasmid pUC18 served as controls. The ethanol values obtained with both sets of controls were essentially the same and were averaged. KO11 (pFGH1A) and KO11 (pFGH12A) were nearly identical (approximately 36.0 g ethanol $liter^{-1}$), providing an increase of approximately 10 % over ethanol production values for the controls containing no plasmid or plasmid pUC18 (Figure 27; Table 8). The addition of KO11 containing pCT603 showed a

more pronounced increase (approximately 20 %) in both the rate of ethanol production and the final ethanol concentration (Figure 27; Table 8). This is the first report of production of 39 g ethanol $liter^{-1}$ with a fungal enzyme loading as low as 5 FPU g^{-1} cellulose.

In conclusion, it appears that the use of bacterial cellulases as a supplement to existing SSF processes with *K. oxytoca* strain P2 as the biocatalyst is an idea worthy of further pursuit. Ethanologenic *E. coli* strain KO11 may be used to generate ethanol from glucose, xylose, or hemicellulose hydrolysate while producing recombinant bacterial cellulase for SSF supplementation. It is possible that a more extensive exploration of preincubation conditions may lead to improvements in ethanol rates or yields. A more fruitful approach may be to try and optimize recombinant cellulase production in *E.coli* strain KO11. Cellulase activity was enhanced when additional ampicillin was added during growth of KO11 containing the cellulase plasmids, presumably due to an increase in plasmid retention. It may be possible to integrate one of the endoglucanases into the chromosome of strain KO11, thereby relieving the problem of plasmid instability. Further studies should help to determine the maximum contribution that bacterial cellulases can make to an SSF process. Using *E. coli* strain KO11 carrying plasmid pCT603, the amount of fungal cellulase required to reach 70% of maximum theoretical ethanol yield was decreased by half.

This value of 40 g ethanol liter^{-1} is regarded by some as a minimum product concentration for cost effective purification (Jeffries, 1988). These experiments suggest that bacterial cellulase supplementation of SSF processes using the recombinant ethanologenic bacteria may help reduce the need for fungal cellulases and possibly reduce the final cost of ethanol to a level that makes it a more economically feasible candidate for alternative fuel production.

CHAPTER VI
GENERAL CONCLUSIONS

Forest and agricultural wastes represent a vast resource for the generation of energy in the form of fuel ethanol. These materials consist primarily of lignified plant cell walls and are composed of cellulose, hemicellulose and lignin. This heterologous polymer represents the dominant form of biomass on earth and a formidable challenge for solubilization and bioconversion. For bioconversion processes, the carbohydrate portion must be solubilized while the lignin and residues can be used to provide energy for ethanol purification (Ingram and Doran, 1994).

The glycosidic linkages in hemicellulose are readily hydrolysed by dilute acids at elevated temperatures to yield a syrup containing xylose and arabinose from agricultural residues and hardwoods, or mannose, xylose, and glucose for softwoods (Puls, 1993). No natural organism has been found which can efficiently and rapidly convert xylose and arabinose to ethanol. However, a series of recombinant bacteria have been developed for this purpose by introducing the *Zymomonas mobilis* genes encoding alcohol dehydrogenase and pyruvate decarboxylase to provide a functional ethanol pathway in enteric bacteria, thus diverting pyruvate metabolism during fermentation from a mixture of acids to ethanol with greater

than 95% of theoretical yield (Ingram et al., 1991; Ohta et al., 1991a; Ohta et al., 1991b). These organisms have been tested on a variety of substrates under many conditions (Barbosa et al., 1992; Beall et al., 1991; Beall et al., 1992; Lawford and Rousseau, 1991; Lawford and Rousseau, 1993; Doran and Ingram, 1993; Doran and Ingram, 1994). There are several areas for incremental improvements in the conversion of hemicellulose-derived sugars into ethanol. The ethanologenic *E. coli* recombinants are fully capable of metabolizing all sugars present at high efficiency to produce ethanol concentrations of at least 50 g liter^{-1}. Thus the current focus and new research must be directed at improving the effectiveness of cellulase enzyme utilization or decreasing the cost of cellulase production.

In addition to the saccharification of hemicellulose, dilute acid treatment serves a second important role in a lignocellulosic conversion process by increasing the accessibility of cellulose to enzymatic attack (Millet et al., 1976; Grohmann, 1993; Grohmann and Himmel, 1991; Converse, 1993). Cellulose is used as an energy source for a variety of microorganisms, including bacteria and fungi occupying diverse habitats. To humans, biomass in the form of cellulosic residues provides a means of harnessing and storing solar energy and thus represents an important energy resource. However, before this renewable carbon source can be used, conversion to a more useful form (glucose, ethanol, or

methane) is required (Kubicek et al., 1993). The saccharification of cellulose is a complex and poorly understood process which involves at least three types of enzymes: endoglucanases, exoglucanases, and ß-glucosidases which together hydrolyse cellobiose and other soluble products to glucose (Enari et al., 1983; Eriksson et al., 1990). Soluble products of these enzymatic reactions act as inhibitors of the saccharification process. To alleviate the inhibition of ß-glucosidases by glucose accumulation, the simultaneous saccharification and fermentation (SSF) process was developed and patented by the Gulf Oil company in which yeasts simultaneously metabolize glucose to ethanol during the saccharification (Blotkamp et al., 1978). Cellulases were supplied as culture filtrates from filamentous fungi such as *Trichoderma reesei*. This patent expired in 1993 and was used for small scale demonstrations, although no large commercial plants were constructed.

Commercially available cellulase preparations from fungi remain too expensive for an economically feasible lignocellulose to ethanol bioconversion process when using these enzymes alone. The cost of cellulase enzymes is a crucial feature for improvement of the economics of ethanol production. One approach to increase the effectiveness of cellulose conversion is to develop recombinant microorganisms which provide some of the enzymes necessary for cellulose hydrolysis, minimizing the accumulation of inhibitory products

and reducing the need for externally supplied fungal enzymes. Using genes from the ethanol pathway of *Z. mobilis*, a recombinant strain of *Klebsiella oxytoca* was generated (Ohta et al., 1991b: Wood and Ingram, 1992). This organism contains a phosphotransferase system for the uptake of cellobiose and cellotriose (Al-Zaag, 1989), thus minimizing the product inhibition of endoglucanases and cellobiohydrolases and eliminating the need for ß-glucosidase.

Studies with highly purified crystalline cellulose as a model substrate indicate that 50% of theoretical yield can be readily achieved during SSF with genetically engineered *K. oxytoca* strain P2 and very low levels of cellulase, 2-5 FPU g^{-1} cellulase (Chapter II; Doran and Ingram, 1993). Precise controls of pH and temperature are not required since relatively broad optima were observed. Under optimal conditions, conversion of cellulose with strain P2 is essentially saturated by 10 FPU g^{-1} cellulose, with only a small gain using 2.5-fold higher enzyme levels. At the lowest level of enzyme, approximately 100 g ethanol are produced per gram of crude cellulase protein.

In SSF studies with sugar cane bagasse, both the rate of ethanol production and yield were limited by saccharification at 10 and 20 FPU g^{-1} acid-treated bagasse. Dilute slurries of biomass were converted to ethanol more efficiently than slurries containing high solids, albeit with the production of lower levels of ethanol. With high solids (ie. 160 g acid-

treated bagasse liter^{-1}), a combination of 20 FPU cellulase g^{-1} bagasse, preincubation under saccharification conditions, and additional grinding (to reduce particle size and facilitate mixing) were required to produce approximately 40 g ethanol liter^{-1}. To improve enzyme utilization a second saccharification step was added after 168 hours of SSF. Almost 40 g ethanol liter^{-1} was produced with 10 FPU cellulase g^{-1} bagasse by incorporating this second step (no further enzyme addition), followed by a second inoculation and short fermentation.

Studies involving the addition of cellulase genes to *K.oxytoca* P2 were unsuccessful due to plasmid instability in this strain. However, SSF supplementation with *E. coli* strain KO11 containing the *celD* gene from *Clostridium thermocellum* showed an increase in ethanol production of over 20% from that of KO11 alone or KO11 containing plasmid pUC18. Since an increase in ethanol production was observed without any optimization of recombinant protein expression in KO11, it may be worthwhile to examine conditions for enhancing this process. Ethanologenic *E. coli* strains contain two foreign genes that have been inserted into their chromosome. There is probably a limit to the amount of additional foreign protein that can be expressed; however it is unclear whether the transformants used in this study approach that limit. It was discouraging to note, however, that there was no obviously overexpressed protein band when French press extracts were

examined by polyacrylamide gel electrophoresis. These extracts did contain CMCase activity as evidenced by the CMCase assays and zymograms.

Cellulolytic enzymes isolated from various sources differ in their molecular characteristics (molecular weight, amino acid composition and sequence, isoelectric point, carbohydrate content) catalytic activity, substrate specificity, and absorbability onto cellulose (Klyosov, 1990). To date, at least 50 cellulolytic enzymes have been cloned and characterized (Beguin, 1990; Gilkes et al., 1991). It may prove productive to screen more of these enzymes for expression in *E. coli* strain KO11. It is possible that one of these previously characterized enzymes can be produced at high levels in KO11. In addition, Healy et al. (1995) have demonstrated that is is not necessary to actually culture an organism in order to clone genes with cellulase activity. Isolation of DNA from the sludge in a thermophilic anaerobic biomass digester yielded several clones with lignocellulose hydrolyzing capabilities. It may be possible to use this approach to isolate total DNA from other sources rich in cellulosic material and discover superior enzymes. Alternatively, there are cellulolytic organisms which can be engineered to produce ethanol as has been done with *Klebsiella oxytoca* or *Escherichia coli*.

In conclusion, there has been much progress made in making the biconversion of lignocellulose to ethanol an

economically feasible process. Many groups are contributing to this goal by generating improved fungal enzyme preparations, by cloning and characterizing cellulase genes, and by improving accessibility of the cellulose by chemical and physical treatments. Contributions are also being made by isolating and characterizing new cellulolytic organisms and by engineering efficient ethanologenic microorganisms. *E. coli* strain KO11 containing recombinant bacterial cellulases provided increases of 10% to 20% over ethanol production values for controls containing plasmid pUC18 or KO11 alone. This is the first report of production of 39 g ethanol $liter^{-1}$ with a low concentration of fungal enzyme (5 FPU g^{-1} cellulose). Additional studies using bacterial cellulases as a supplement to existing SSF processes may allow further reduction in the amount of fungal cellulase enzymes needed for adequate ethanol production and may help speed the conversion process.

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BIOGRAPHICAL SKETCH

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